XVIth International Congress on Animal Hygiene

"Animal Hygiene, Health and Welfare as Corner Stones of Sustainable Animal Production"

May 5–9, 2013 Nanjing, China

ISAH 2013

XVI[th] International Congress on Animal Hygiene (ISAH-2013)

ISAH 2013

INTERNATIONAL SOCIETY FOR ANIMAL HYGIENE

ISAH

ANIMAL HEALTH & WELFARE
BIOSECURITY
ENVIRONMENTAL PROTECTION
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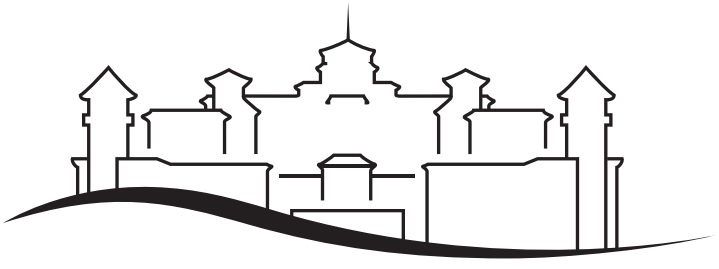
XVIth ISAH 2013 Congress

Proceeding of the XVIth International Congress of the
International Society for Animal Hygiene

"Animal Hygiene, Health and Welfare as Corner Stones of Sustainable Animal Production"



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Preface

It is our greatest honor and pleasure to organize first time the XVIth Congress of ISAH 2013 in Nanjing, China. On behalf of the Organization Committee, the Scientific Committee and the Executive Board of International Society for Animal Hygiene (ISAH), I cordially welcome you in Nanjing, China to participate in the XVIth Congress of ISAH 2013.

The bi-annual congresses of ISAH initiated in 1970 have been identified as an influential conference worldwide for professionals in animal hygiene, health and welfare to discuss and develop the latest research breakthroughs. ISAH is an association of veterinarians and other professional scientists, practitioners and students working in the field of animal health and welfare, animal hygiene, biosecurity, safety of animal-origin food, environmental protection in relation to animal production and related areas. ISAH is a high-profile international organization with members in 57 countries and areas all over the world.

Animal hygiene encompasses the knowledge of all relevant aspects of the abiotic and biotic environment of animals in a housing and keeping system and the understanding of the principles of the interactions of animals with their technical, physical (thermal), chemical and biological surrounding in order to improve their health, welfare and performance without the unnecessary use of drugs being the preconditions for the production of safe and healthy food. Furthermore, it requires a profound knowledge of management, handling and behaviour of animals on individual and herd level, and the understanding of the effects of animal husbandry systems on the surrounding environment, such as airborne emissions, manure disposal and spread of diseases (environmental hygiene), in order to define prevention strategies including bio-security measures and disinfection. The concept of hygiene integrates in this way the relevant aspects of animal health and welfare in the livestock production, ethics, ecology and consumers demands on a sound economic basis.

The motto of XVIth Congress of ISAH 2013 is "**Animal Hygiene, Health and Welfare as Corner Stones of Sustainable Animal Production**" and the scientific programme follows the scope of the ISAH and concentrates on the following subjects: Animal health, animal welfare and behaviour; Hygiene in animal production, disinfection; Preventive veterinary medicine and herd/flock health management; Veterinary public health and food hygiene; Zoonotic disease and prevention; Climate change and emerging diseases; Feed and water quality; Feed additives and nutrition; Environmental pollution, emissions and abatement options; Waste management and biogas production; Precision livestock farming (PLF); Food hygiene; Basic and biomedical science.

The Proceeding from the XVIth ISAH 2013 Congress presents papers of lectures from invited keynote speakers, oral and poster presentations held in 4 parallel sessions. The organization of such a congress requires the help and input of many people, and I hereby would like to express my deep thanks to all who contributed to realize XVIth ISAH 2013. My most sincere gratitude goes to ISAH 2013 Organization Committee, the Scientific Committee and the Executive Board of ISAH. Our gratitude also goes to Chinese Association of Animal Science & Veterinary Medicine (CAAV). Big thanks go also to all our other supporters from industry and business and all those that opened their premises for our technical tours.

My special thanks are reserved to our host, Nanjing Agricultural University (NAU) and the involved university services. I would like to thank all the many helping hands, most prominently the competent experts, president, Prof. Guanghong Zhou, and vice-presidents, Prof. Qirong Shen, Prof. Jianjun Dai, of NAU; Prof. Hongsheng Zhang, the director of Foreign Affairs of NAU; Prof. Hongjie Fan, the dean of College of Veterinary Medicine; Prof. Honglin Liu, the dean of College of Animal Husbantry and Technology, for their invaluable help and support. Big thanks go also to Ms. Aihong Xia and Ms. Bo Shen for their excellent and indefatigable editing work. Special appreciation and big thanks go also to Prof. Hartung, Ms. Petra Sommer at University of Veterinary Medicine Hanover, Foundation, Germany for their contributed ideas and continual support.

Last but not least, it is my privilege to thank all participants, contributors, chairpersons, organizational and technical assistants for their considerable efforts and inputs. We wish you all have a meaningful and pleasant XVIth ISAH 2013 Congress and an enjoyable time during stay in Nanjing, China.

Prof. Dr. Endong Bao
The 2nd-vice president of the ISAH 2013 Organization Committee

List of ISAH Congress

ISAH founded 20th November 1970 in Budapest, Hungary

	Year	Place of Venue	Organizer
I	Congress 1973	Budapest, Hungary	F. Kovacs
II	Congress 1976	Zagreb, Yugoslavia	J. Ivos
III	Congress 1980	Vienna, Austria	H. Willinger
IV	Congress 1982	Strbske Pleso, Czechoslovakia	J. Rosocha
V	Congress 1985	Hannover, Germany	H. G. Hilliger
VI	Congress 1988	Skara, Sweden	I. Ekesbo
VII	Congress 1991	Leipzig. Germany	G. Mehihorn
VIII	Congress 1994	St. Paul, Minnessotta, U. S. A	S. L. Diesch
IX	Congress 1997	Helsinki, Finland	H. Saloniemi
X	Congress 2000	Massstricht, Netherlands	M. Tielen
XI	Congress 2003	Mexico City, Mexico	J. A. Saltijeral
XII	Congress 2005	Warszawa, Poland	A. Krynski
XIII	Congress 2007	Tartu, Estonia	A. Aland
XIV	Congress 2009	Vechta, Germany	J. Hartung
XV	Congress 2011	Viena, Austria	H. Schobesberger
XVI	Congress 2013	Nanjing, China	Endong Bao

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Waste Management and Biogas Production

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Food Hygiene

Basic and Biomedical science

General Topics

Poster

Talk of Keynote Speaker

Emerging and Re-emerging Diseases—The Role of International Organisations

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Abstract: Emerging diseases are new infections resulting from the evolution or change of an existing pathogen or parasite resulting in a change of host range, vector, pathogenicity or strain, Prominent examples are the influenza epidemics in the last 10 years: H5N1, H1N1, and brand new, the Avian Influenza H7N9, but also not to forget HIV, SARS and the Schmallenberg virus. They have very often an animal origin and almost all of them have zoonotic potential.

Re-emerging diseases are considered already known; they either shift their geographical setting, or expand their host range, or significantly increase their prevalence in a specific region. Actual examples for re-emerging diseases are Bluetongue in North-western Europe, West-Nile Fever in Northern America, and PPR in Africa.

The rapid detection and response to an emerging or re-emerging disease is crucial. The rapid detection of such a new epidemiological event is therefore a key element for all policies to be developed. With globalisation and the increase in speed and volume of international transport as well as passengers travel, the new pathogens begin their global spread. All depend therefore of the availability of a functioning veterinary infrastructure, expertise, diagnostic laboratories and surveillance capabilities as a whole. Unfortunately many countries are still ill equipped in this respect, although huge efforts have been made during the global H5N1 epidemic.

International Organisations such as the World Organisation for Animal Health (OIE), FAO and WHO have started a closer cooperation to address the new challenges and adopted in 2010 a Tripartite Concept Note addressing animal and public health risks attributable to zoonoses and animal diseases with an impact on food security, through multi-sectoral cooperation and strong partnerships. Animal Influenza, rabies and anti-microbial resistance have been identified as priority topics during an Inter-ministerial high level meeting, held in 2011 in Mexicao City.

According to its main mission, the OIE is supporting Veterinary Services worldwide through the PVS-tool, assisting countries in determining their current level of performance and compliance with the OIE-standards on the quality of Veterinary Services. Together with WHO the OIE is currently developing a OIE-PVS "One Health" tool, aiming at the evaluation of the level of cooperation between Veterinary Services and Public Health Sevices, as well as with other relevant stakeholders at the animal-human interface. Although not yet officially recognised by WHO and OIE Members, pilot missions have been deployed to test this new tool.

The talk will describe specifically the different missions and activities of OIE directed to improve early detection and control of emerging and re-emerging diseases.

Precision Livestock Farming as a Tool to Improve the Welfare and Health of Farm Animals

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Summary: The worldwide demand for meat and animal products is expected to increase by at least 40% in the next 15 years. The first question is how to achieve high-quality, sustainable and safe meat production that can meet this demand. At the same time, livestock production is currently facing serious problems. Concerns about animal health in relation to food safety and human health are increasing all the time. Europe wants improved animal welfare and has made a significant investment in it. At the same time, the environmental impact of the livestock sector is far from being solved. Finally we must ask how the farmer, who is the central figure in this process, will make a living from more sustainable livestock production. One tool that might provide real opportunities is Precision Livestock Farming. Continuous, fully automatic monitoring and improvement of animal health, welfare, yields and the environmental impact should become possible. This paper presents examples of systems that have already been developed in order to demonstrate the potential of this technology.

Problem

All published forecasts and predictions expect the *worldwide demand for meat* to increase by at least 40% in the next 15 years (FAO, 2007).

At the same time the world is worried about disease transfer from livestock to humans and this makes *animal health* a top priority. There is significant room for improvement in the treatment of health problems on farms; for example use of antibiotics is far too high and must be reduced.

Europe has recently invested a lot of money in developing a methodology to score *animal welfare on farms*. The EU now wants to implement this in practice by means of new directives. Farmers, however, are already squeezed by so many rules and laws that this is not an attractive task for an independent entrepreneur.

Finally, we must find a means of implementing more sustainable livestock production systems as the *environmental impact* of our meat production system is currently too high. For example, more than 92% of the ammonia in the environment is due to animal production. In naturally ventilated buildings there is no accurate way of measuring the ventilation rate and consequently no accurate way of measuring gas emissions. Most of the livestock houses worldwide are naturally ventilated houses.

As a result of all these factors, it is no longer easy to make a living from livestock farming. Farmers have to manage a complex situation in balancing feed and energy costs against meat prices. The animal is influenced by many physical and social/environmental variables that must be controlled in order to manage a number of process outputs such as animal health and welfare, product quality, environmental impact and, last but not least, a decent economic return for the farmer.

Worldwide demand for animal products is increasing, but at the same time the number of farmers producing animals is decreasing year on year. As a consequence livestock farms will not become smaller but will continue to increase in size. Today the profit per animal is so low that the farmer needs to keep more animals in order to earn a decent income. As a result, he has *less time available per individual animal*, which makes it more difficult to monitor and manage his animals properly. This is a key problem: the modern farmer is too far away from the essential part of this biological process - the animal. He has to take more decisions sitting at his computer as he cannot spend enough time in contact with his animals.

Due to the economic constraints, it is unlikely that the number of animals on a farm will decrease. Even small farms with a limited number of animals must deal with problems such as product quality control, animal health and welfare and bio-security. Many stakeholders are involved in livestock production nowadays; these include the animal, the farmer, retailers, the equipment industry, consumers, the general public, the press and governments. For most of them it is clear that a new approach is needed if we are to solve the problems we are currently facing in livestock production. Wherever in the world this process takes place, farmers face the question

of how to incorporate all the requirements for all the individual living organisms into a sustainable livestock production system.

Precision livestock farming: biology meets technology

The objective of Precision Livestock Farming is to create a *management system based on continuous automatic real-time monitoring and control of production/reproduction, animal health and welfare, and the environmental impact of livestock production.*

Precision Livestock Farming (PLF) is based on the assumption that continuous direct monitoring or observation of animals is crucial in enabling farmers to detect and control their health and welfare status at any given time. Ultimately, a happy and healthy animal might provide the best guarantee of product quality in the long term. Nowadays the farmer can use modern technologies to measure different parameters on the farm (ventilation rate, feed supply, heating/cooling supply, etc.), but none of the tools available up to now has measured the most important variable in the production process: the animal.

Technological development and progress have advanced to such an extent that accurate and affordable tools are now available: cheap and powerful cameras, microphones, sensors (3D accelerometers including gyroscopes, temperature sensors, skin conductivity sensors, glucose sensors, etc.), wireless communication tools, internet connections, cloud storage, etc. Modern technology makes it possible to place cameras, microphones and sensors so close to the animal that they can replace the farmers' eyes and ears in monitoring individual animals.

The aim of PLF is to combine all the available hardware with intelligent software in order to extract information from all the data. PLF can offer a management tool that enables a farmer to monitor his animals automatically and to create added value by guaranteeing improved health, welfare, yields and environmental impact.

Examples of monitoring by PLF

Continuous health monitoring by real-time sound analysis

Respiratory pathologies are widespread in intensive pig farms; their incidence and prevalence are high and their principal symptom is coughing. The importance of these diseases must be viewed from both an economic and a hygiene perspective in terms of the high cost of veterinary intervention and the loss of profit due to higher mortality (in 15% of cases) (Baumann, 2002) or a drop in production due to reduced feed conversion and a lower growth rate. It is very unlikely that a pig will reach the slaughter weight without having suffered some kind of respiratory problem (Leman, 1992). It is also known that detecting illness in individual animals and providing individual care or group-by-group mass treatment in response to illness are not very effective, and are also costly. It is crucial to investigate cough sounds with the aim of understanding respiratory diseases and to use bioacoustics for real-time monitoring purposes.

The importance of coughing as a means of prediction tool applies to animals as well as to humans. It has been shown that pig vocalisation is directly related to pain and classification of such sounds has been attempted (Marx, 2003). It is also common practice among veterinarians to assess cough sounds in pig houses for diagnostic purposes. In this regard, there have been attempts to identify the characteristics of coughing in animals (Moreaux, 1999; Van Hirtum, 2002a) and to automatically identify cough sounds from field recordings (Van Hirtum, 2002b; Van Hirtum, 2003).

Since the sound produced while coughing has special characteristics when the respiratory system is infected, the possibility of "labelling" the different sounds as soon as possible can be a very useful instrument for prompt disease detection so that targeted interventions can be prepared (isolation of the box, targeted use of antibiotics, etc.).

Algorithms to distinguish between pig coughs have already been validated. Initially, these algorithms were able to distinguish between sounds, then between coughs and sounds, and more recently between pathological and non-pathological coughs. (Van Hirtum et al., 1999; Chedad et al., 2001, Van Hirtum and Berckmans, 2002) (Fig. 1).

Validation of these algorithms in the field, conducted in a piggery in Lombardy (Italy), has shown that the algorithms developed are able to classify the cough correctly in 86% of cases (Guarino et al., 2004).

Continuous welfare monitoring by real-time image analysis

By introducing cameras into livestock houses it is possible to monitor the animals at an image speed up to 25 images per second using normal cameras. When top view cameras are used it is possible to develop many different algorithms that are easy to implement in real livestock houses.

In broilers, for example, the eYenamic system has been developed for continuous automatic monitoring of broiler behaviour in a livestock house. The EU specifies that a broiler farmer must carry out a visual inspection of his animals at least twice a day, but the eYenamic system does the job continuously. The zone occupation index and

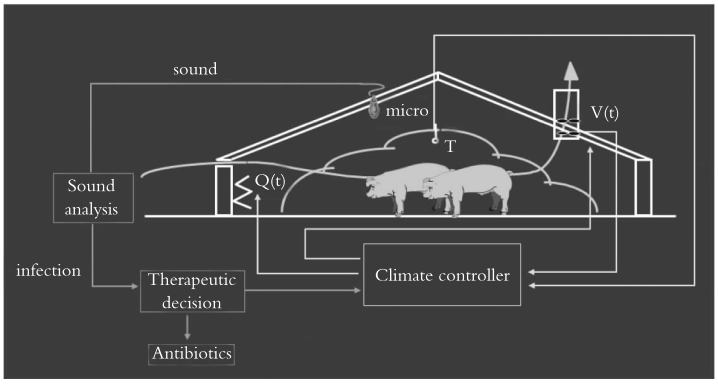

Fig. 1 Sound analysis is used in real time to monitor the health status of pigs

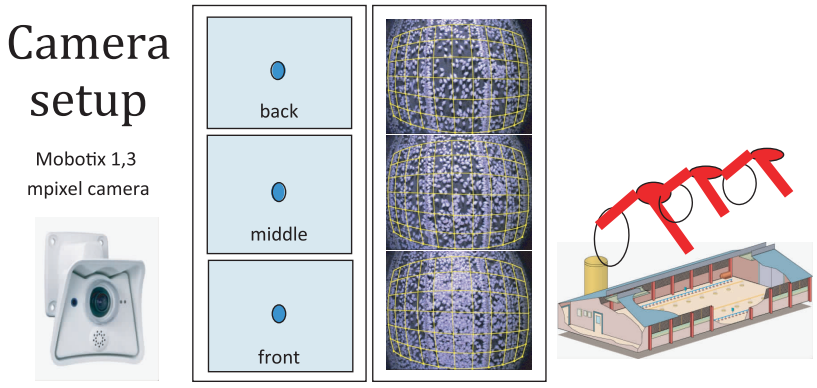

Fig. 2 The eYenamic camera system for monitoring broiler behaviour

zone activity index of the broilers is calculated from the top view cameras in the broiler house (Fig. 2). As shown in Fig. 3, the zone occupation index of the birds can vary greatly from 15% at 02.00 hours to 50% in the afternoon when the inside temperature is higher in that specific zone. The 15% zone occupation index shows that birds only occupy that zone so that they are close to the drinking line for eating or the feeder pans, indicating that something might be wrong with the temperature or air flow pattern. If the system generates abnormal values, it immediately sends the farmer an alarm message and advises him to check what is wrong with the animals.

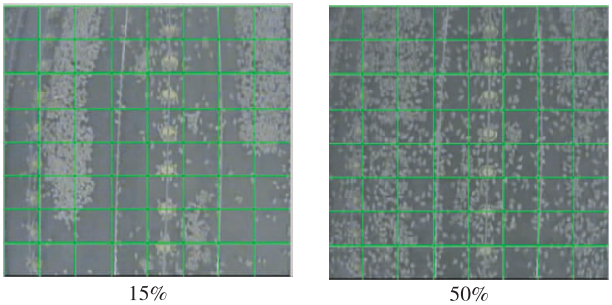

Fig. 3 Images used to calculate animal activity, zone occupation index and animal distribution

Another interesting example is automatic camera analysis of dairy cows whenever they approach the milking robot. By carrying out image analysis and calculating model parameters from the image information, it was possible to develop an algorithm for automatic detection of lameness problems in dairy cows. Such techniques provide continuous and fully automatic monitoring of all individual cows, a process which the farmer can no longer carry out himself(Fig. 4).

Controlling processes using PLF tools

By carrying out real-time measurements and developing real-time models it becomes possible to apply the global concept as explained in Fig. 5. When the weight of growing broilers is measured as a process output and the amount of feed delivered is also measured, it is possible to model the dynamic response of weight to feed supply (Fig. 5).

The computer calculates this relationship every day and the relationship obtained from the results for the last 5 days will predict the expected variation for the next day. The fact that we can now obtain daily information about how the weight of a given flock of broilers will respond to a given feed supply makes it possible to calculate the daily feed requirement that will enable those

Fig. 4 Real time image-based model to calculate gait parameters for lameness detection

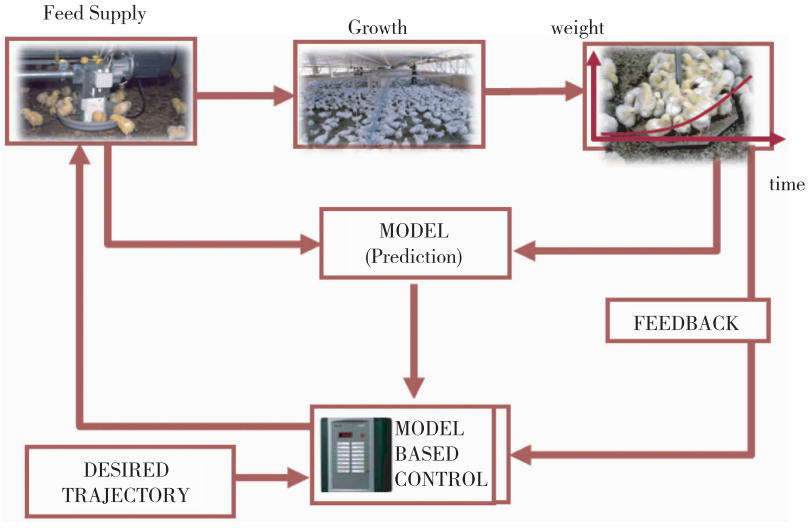

Fig. 5 The basic PLF scheme of Fig. 3 applied to monitoring and control of broiler growth using feed supply as the control input (Aerts et al., 3003)

broilers to follow a specific growth curve. Fig. 6 shows how this approach enables broilers to achieve a predefined growth trajectory which is completely different from the control group with ad lib feeding. The predefined target line could be achieved quite accurately and had the advantage that bird growth was initially slower, which allowed the bone structure to develop better. From a certain point in time, controlled growth increases to reach a similar end weight but with significantly fewer leg problems and less mortality.

Technology is ready to meet biology

Fortunately the *new technology* is now reaching the point where its application to biological processes becomes realistic. Wireless data transmission, for example, is becoming cheap and reliable. The sensor and sensing (camera, sound) technologies that are needed to develop Precision Livestock Farming (PLF) products, have become small and reliable enough to be used within the harsh environment of livestock production.

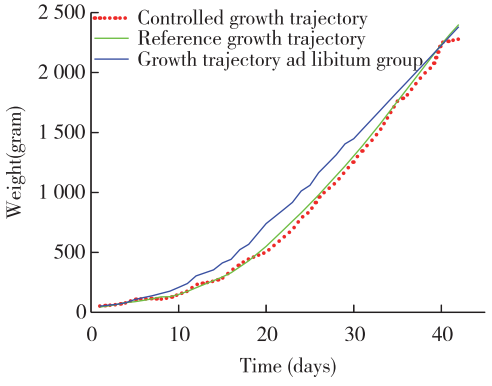

Fig. 6 Resulting growth trajectory (dotted line) of broilers following the target line and the difference from the growth trajectory of ad libitum fed broilers (Cangar et al, 2007)

Within technology development there are some important rules and milestones which speed up both application of the technology and progress. One of these

important rules is that the main factor determining the cost of technology is the number of units produced. The fact that devices such as mobile phones are so successful worldwide has an important impact on the decreasing cost of wireless communication and is pushing this technology into other applications such as PLF. Moreover, the livestock market involves huge numbers of animals and processes, making it possible to produce customised, applied technology at reduced costs.

Conclusion

If properly applied, Precision Livestock Farming will offer new opportunities to increase the efficiency and sustainability of farming and livestock production, to improve the health and welfare of animals and to guarantee full traceability of the entire supply chain in order to provide the consumer with a guarantee of food safety.

New milestones could be reached in a short time, but if we are to achieve results quickly, a number of disciplines will have to come together and create new synergies in order to provide space and opportunities for a wide range of skills (veterinary, agricultural, engineering, ethological, medical, physical, mathematical, etc.).

PLF technology will create added value for many stakeholders, especially as a management tool for farmers, making it possible to improve animal welfare, health, efficiency and the environmental impact.

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Management of Animal Hygiene as an Important Component of Food Safety Control Strategies

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Summary: Food safety has become one of the key issues in public health management with increasing concerns from the general public around the world. Despite significant efforts in good manufacturing practice and HACCP at the processing and retain levels, there are still a considerable number of outbreaks of foodborne illness not only in developing countries, but also in developed countries mostly due to microbial pathogens. It seems that the food safety challenges of more than 20 years ago still persist[1] Furthermore, the burden and cost of foodborne diseases associated with microbial pathogens is largely unknown for most countries. WHO Initiative to estimate the global burden of foodborne diseases aims to fill the current data gap that are important for policy-makers to prioritize resources to address the major problems [2]. A recent US study shows that the annual cost of foodborne illness could be 51 billion US dollars in average ($ 31.2 – $ 76.1 billion) with mean cost per case at $ 1626 based on 2011 estimated 48 million cases [3]. In Australia, the estimated yearly cost for foodborne diseases was A $ 1.2 billion for around 5.1 million of cases per year [4].

Foodborne illness due to transmission of pathogens from primary production

Microbes can enter the food chain at different steps along the food chain from primary production, are highly versatile and can adapt to the environment allowing survival, growth and production of toxins. Systemic information on microbial foodborne illness is largely from developed countries where active surveillance programs have been in place. In the US, foodborne salmonellosis and campylobacteriosis were on parallel top list in 2011, 16.4 and 14.3, respectively per 100,000 population. Of the 45 outbreaks of salmonellosis since 2006 in the US, food of animal origin and fresh produces were both implicated [5]. In Europe, campylobacteriosis has been ranked No. 1 for since 2006, and poultry meat was the major contributor [6]. Cantaloupe was found as the source of a multi-state outbreak of listeriosis with 147 cases and 33 deaths [7].

In developed countries where good hygiene practice or HACCP has been implemented in the food production systems, continuing threat of microbial foodborne illness suggests failure mechanisms of current measures in preventing microbial contamination of foods. Primary production has been increasingly implicated as sources of some foodborne outbreaks that could be directly traced back to raw animal products like raw milk, pork and eggs [8 – 10]. A recent study reveals that hepatitis E virus could be recovered from pork liver sausage [11], most likely carried over from live pigs. Pork is the predominant source of outbreaks of human trichinellosis in China [12]. Much research into food-borne human pathogens has focused on transmission from foods of animal origin. However, recent investigations have identified fruits and vegetables are the source of many disease outbreaks [5, 7, 13]. Severe outbreaks of *E. coli* O157:H7 (or even O104:H4 occurred in Germany and other parts of Europe in 2011) were traced to consumption of contaminated sprouts and pre-packaged spinach [11], although their infections have been linked to beef more often than to any other food product. It is now clear that primary production of both food animals and vegetables or even fruits contribute to foodborne illness.

However, there appear to be of no efforts to track down the source of microbial contamination of vegetables. An apparent question is: Are they related to animal manure used as fertilizers? A better understanding of the factors that facilitate microbiological transmission through the environments in the animal-plant interface would help develop evidence-based policies and technologies aimed at reducing the risk of contamination of fresh produce that are to be consumed unprocessed as salads.

Preharvest control of major foodborne pathogens in primary production

With continuing outbreaks of foodborne illness back in 1990s irrespective of control measures in place in the processing industries, the significance of controlling hazards along the food chain from the farm level has been recognized [14]. In Europe, agreement has not been reached on practical systems of HACCP relevant to food safety during primary livestock production. The EU

hygiene package (Regulation (EC) No 853/2004) recommends exploration of the feasibility of the application of HACCP during primary production. A good example is the pathogen reduction HACCP program implemented by the USDA's Food Safety and Inspection Service (FSIS). This program consisted of a staged implementation between 1996 and 2000 to reduce microbial contamination on meat and poultry products. Efforts continued as shown by the US National Institute of Food and Agriculture funded Preharvest food safety grants of about 70 million USD from 2005 – 2010 for research on improving measures for increasing the safety of food of animal origin: reduction, prevention, control and surveillance of risks in farm animal production.

Of the commodities regulated by FSIS, one of the largest observed effects was reduction of *Salmonella* contamination on broiler chicken carcasses. A recent modeling study shows that nearly 190,000 fewer annual salmonellosis cases (attributed to broilers) occurred in 2000 compared with 1995, following the 56% reduction in the proportion of contaminated broiler carcasses observed between 1995 and 2000 (with the uncertainty bounds for this estimate range from approximately 37,000 to 500,000 illnesses). However, estimated illnesses prevented, due to the more modest reduction in contamination of 13% between 2000 and 2007, were not statistically significant [15]. A European report indicates that the public health benefits of controlling Campylobacter in primary broiler production are expected to be greater than control later in the processing chain since the bacteria could transmit from farms to humans by routes other than broiler meat [16]. Strict implementation of biosecurity in primary production and GMP/HACCP during slaughter may reduce colonization of broilers with Campylobacter, and contamination of carcasses. However, it is suggested that the effects could not be quantified because of confounding factors in different settings.

In developing countries including China, HACCP is not mandatory in processing plants, let alone its implementation in the primary production. More importantly, there is serious information gap on the outbreaks of foodborne illness attributable to prevalence of pathogens in the primary production of food animals and vegetables. There were quite a lot of publications that report prevalence of major microbial pathogens in processed food and raw meats, milk or eggs. No report exists on vertical epidemiological analysis of potential pathogens at the molecular level along the food chain from primary production. Lack of such important information would seriously jeopardize the process of risk assessment and management aimed at reducing public health consequences due to transmission of pathogens into food chain from the farms.

Future perspectives in preharvest food safety research and management

The risk factors that contribute to microbial food safety could differ among different countries because of the differences of dinning habits and catering as well as differences of husbandry and/or agricultural practices. With globalization, diet culture has become mixed. For instance, fast food restaurants or ready-to-eat food has increasingly become part of the ordinary life in Asian countries like China. Therefore, the food safety problems in these countries could be more complicated, though not necessarily significant in prevalence.

In developing countries where baseline data on prevalence and burden are largely unknown, efforts should be made to set up active surveillance programs and to conduct risk assessment in relation to the categories of food implicated as well as the types and sources of microbial pathogens in order to prioritize the foodborne illness control strategies at different steps of food production including primary production on the farm. This is to fill the knowledge gap in prevalence of major pathogens in the primary production sites and their contribution to foodborne illness. Attention should be paid to ensure that the quality of investigation be scientifically sound for meta-analysis from national or global perspectives [17].

Although the preharvest food safety research in developed countries has led to better understanding of the importance of good agricultural practice in ensuring safer food, the eco-epidemiological aspects of major foodborne pathogens, including potential zoonotic viruses such as hepatitis E virus, at the animal-plant interface or more broadly at the animal-plant-human interface should be explored in more detail to track down the source of contamination of fresh produces implicated in foodborne illness. This is now made possible by using molecular fingerprinting techniques such as multilocus sequence typing (MLST) to trace the transmission dynamics of pathogenic organisms [18].

Another area of interests is the cost-benefit analysis of preharvest practices that are additional to animal health management and mainly targeted for food safety purposes. In Denmark, it was shown that control of Salmonella in pigs and chicken was cost-effective in compensating for public health economy [19]. A US study shows that changes in Salmonella status during processing such as rinsing carcasses at various temperatures with and without sanitizer are more important for human health risk and have a higher benefit:cost ratio when compared with on-farm strategies for Salmonella control including vaccination and meal feeding [20].

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Gut Microbiota: Bioconversion and Metabolism of Nutrients and Non-nutritive Bioactive Compounds

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Abstract: The gastrointestinal tract of the animal harbors dense and highly complex community of microorganisms composed mainly bacteria. These bacteria have potential effects on host nutrition and health through the conversion and metabolism of dietary components including nutrients and non-nutritive bioactive compounds, with the metabolites in turn nutritively more available or affecting epithelial barrier function.

The small intestine is one of the most important sites for digestion and absorption of nutrients. Meantime, considerable proportions of nutrients are extracted by the small intestine during the first-pass intestinal metabolism especially for amino acids (AA). While the small intestinal epithelial cells lack enzymes to catabolize essential AA, the fate of non-absorbable AA remains unclear. Recent studies have suggested that small intestinal bacteria may contribute to the host's metabolism amino acids. This paper will summarize our recent research progress on the utilization and metabolism of individual AA by the pig small intestinal bacteria. We used pig as the animal model to characterize the utilization and metabolism of amino acids by small intestinal bacteria *in vitro*, to analysis and identification of the bacterial populations associated with the utilization and metabolism of individual amino acid, and to evaluate the effects of amino acid supplementations (e.g., glutamine and arginine) on the utilization and metabolism of amino acids by small intestinal bacteria.

In vitro incubation, subculture, metabolites analysis technique, and radioactive isotope tracer technique were used to investigate the utilization and metabolism of amino acid in pure bacterial strains and mixed bacterial cultures derived from different compartment of the pig small intestine. Further, the 16S rRNA gene based molecular techniques were employed to analysis and identification of the bacterial population associate with the metabolism of individual amino acids. We also evaluate effects of increased dose of glutamine or arginine on the amino acid utilization and metabolism in pig small intestinal bacteria. The observations from our study indicated that: 1) the small intestinal microbiota was active in the compartmental metabolism of specific groups of dietary AA in the pig small intestine; 2) the structures of the bacterial population associated with the metabolism of individual AA in the small intestine might be AA-dependent yet share the common core and 3) supplementation of glutamine or arginine modulates the metabolic fluxes of AA and its metabolites in the AA metabolic networks in pig small intestinal microbiota and the regulatory effect is bacterial species and gut compartment specific. The findings from this study might provide the frameworks for further *in vivo* investigations and help to develop strategies in the modulation of the microbiota composition and activity associate with the metabolism of proteins and amino acids in the small intestine aiming at improving the nutrition and health of both humans and animals.

The intestinal microbiota play important roles in the conversion of the bioactive compounds in the gut. Plant derived bioactive compounds such as flavones and isoflavones have become increasingly important in animal production at the time of banning of antibiotics as feed additives. However, the biological activities of these bioactive compounds depend on their bioavailability. Metabolism by the intestinal microbiota is an important factor for the bioavailability of the bioactive compounds. We have isolated a number of bacteria capable of bioconversion of isoflavones (daidzein) and flavonoids (hesperidin and genistein) from the intestine of pig and chicken. Equol production in pigs (sows and piglets) may be related to a specific group of microbial community. The bioavailability differed for the compounds depending on the structure and glycosidic form. Organic acids may reduce the bioavailability of flavonoids while FOS may help in preserving the deglycosylated metabolites depending on the subclass of flavonoids. Dietary supplementation of flavonoids (hesperidin and genistein) could improve performance and health of broiler chickens by stimulating the immune systems. The results suggest that the effectiveness of plant bioactive compounds may depend on the bioconversion and production of active metabolites by the intestinal bacteria and that a dietary intervention using a combination of plant bioactive compounds and their metabolizing bacteria may provide a potential strategy for improving animal performance and health status.

Animal Welfare, Health and Behaviour

The Differences with Canibalismus in Pig Farms in China and Germany

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Summary: In Germany we have problems with canibalismus and tail biting. During my farm visits in China I do not found these problems with canibalismus. There are differences in pig farming in these two countries, as there are other races, other feeding systems, other groups and contact with the pigs during the day.

Introduction

Since many years the PR China is a member of international organisations as the "World Organisation for Animal Health" (OIE). There constitution determins, the veterinarian has to "promote the animal health and welfare". The Europeans have since many years a law for protection of all animals and they have special rules for house hold animals as dogs, cats, pigs, cows, poultry etc.. In the EU regulation 2008/120/EG and in the Cross Compliance rule VO EG No. 73/2009 we find standards for protection pigs in farms. In the PR China till now there are only protection rules for wild animals as the "panda" (ursidae).

Material and methods

During the visits in the private pig farms with sows and fatteners in Germany by the animal health service (SGD) and in China by the Senior Expert Service (SES) in pig farms from 50 to 1000 sows and stables for fatteners in different chinese provinces we watched the housing places, overstocking, ventilation, nutrition, genetic, canibalismen etc.. All these dates and facts were protocolled and used for determine the differences in pig housing, management and canibalismus in this two countries.

Results

During the visits in the pig farms in Germany and in China we found differences in pig farming, housing, farm ventilation, feeding and management. In Germany there are many problems with canibalismus and tail biting. In the tables 1 and 2 there are the results of the differences in farm management in the two countries. In Germany we have other races with more biting, automatic ventilation systems, more mixing of piglets and fatteners with piglets from two or three farms. In Germany there are in one pen 40 to 100 piglets from three or five different sows. In the chinese pig farms we have found in one pen only 20 piglets from two sows. By the automatic feeding and manure systems in Germany the farmers have only time for one short visit a day with the pigs. In China much

Table 1 Management, farming, races differences in pig farms Germany and China

Parameter	Germany	China
canibalismen	frequent	rare
season	autumn, winter, spring	winter
pig tail resection	yes	not
tail biting	frequent	rare
biten pigs	runt disease	angry
biting pigs	femal, older, sickly	aggressive
social ranking	high	low
temperament	high	low
lighting	day-, artificial light	daylight
races	hybrid	LW × Duroc
meat-feat-relation	meat: much, feat: low	meat: low, feat: high
back feat	low	high
stocking density	high	low
nest box	open	closed
pig cluster	high	low
human contact	low	high
delivery farms for pigs	different	one

Tabele 2 Management, housing, climate differences in pig farms Germany and China

Parameter	Germany	China
change of housing	more	two
feeding technic	automatic	per hand
feeding composition	soja, harvest, traceelements, vit.	soja, cornmais, traceelements, vit.
structure	low	high
mycotoxine	low	high
busy material	yes	no
contact to farmer	low	high
air condition	automatic	natural
air gases	CO_2: high, NH_3: high	low
housing temperature	automatic regulation	natural with windows
housing floor	slatted concret floor	slatted grate, concrete floor

more workers as in Germany have a job for feeding and cleaning in the pig stables. The german pig farms with places for 500 sows and stables for 1000 fatteners have only three workers and the farms in China have 20 to 30 workers for the stables. During a day the Chinese workers are very often in the stables together with the pigs for controlling, feeding, cleaning etc., as the most farms do not use so much technical equipment as in Germany.

Discussion

In Germany and other countries in the world canibalismus and tail biting is a problem with reduced weight gain, infections, runt diseases and losses during fattening. Pigs have a natural routing instinct. If they are not eating, drinking and sleeping they are looking around in the pen for doing something. In the modern stables we keep the pigs overcrowded and in unnatural conditions [1]. Till now in China the races (LW × Duroc) for fattening are quite and not so aggressive. They are in pens with maximum 20 animals, have intensive contact with each other and with the farm workers. They have a low stress level during there live. For more then 80% of the day they are dozing together. In China with the natural air in the stables there are not so much toxic gases during the year as in Germany. There have a higher welfare with a reduced aggression. In a check list (Table 3) for tail biting are written the different factors for this problem. Overstocking, ventilation, air speed and cold draughts, sick pigs and boredom are the important facts for tail biting[2].

Table 3 Tail biting checklist with main areas to be examined and rating importance

Areas of attention	Rating importance(%)
overstocking	60
ventilation inadequate	50
gases CO_2, NH_3	15
low speed, cold draughts	40
bady placed furniture causing agression	10
uneven mixing	18
pigs moved off bedding (straw) to slat floors	20
poor trough design, inadequate feeder space	15
sick pigs not removed promptly	60
genetic: aggressive	15
nutrition: Mg, Ca	20
mycotoxin	15
water in adequacy	20
boredom	50

Conclusions

During my visits in pig farms in Germany and in China I found differences in pig farming in this two countries. In Germany the farmers have problems with tail biting. There are other races, more technical equipment in the stables, big fattening groups and the farm workers have minimal contact with the pigs during the day. The pigs in Germany are often overcrowded and have more stress then in Chinese pig farms.

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Acupuncture and Horses Medicine between Ancient Arabs and Chinese

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Summary: The animals were very important in ancient Arabs especially horses, because the horse was very important for their life in desert and for defense against enemies of trip, after declaration of Islam increased needs for animals, added a relations with neighbours Greek, Indian, Persian, all these factors reacted with us and resulted in development of ancient Islamic Arabic veterinary medicine. Aquapuncture may be defined as the insertion of needles into specific points on the body to cause a desired healing effect but in ancient bloodletting and cauterization were included. Many of veterinary techniques these Chinese source describe using on horse correspond closely to techniques found at the same time or even earlier in Egyptian, Arabic, Indian, and other sources. Herbal medicine for horse was found in ancient Arabs, Chinese and other ancient civilizations. Cauterization is one of the oldest means of treatment in the world, by glowing iron rods local different degree of burning marks are made in the skin, The site of application vary for different diseases, generally the location is anatomically related to the sites of the illness, the Arabs medicine has influenced the European and other countries in the medieval times and cauterization became an important tool in the treatment of mankind and animals, nowadays cauterization is still practiced in folk medicine all over the world. Chinese veterinary medicine of horses is back to long periods, it revolves around the diet, exercise, and environment of horse, it also involve the use of Aquapuncture and Chinese medical herbs.
Key words: history of veterinary medicine, Arabic veterinary medicine, Chinese veterinary medicine, acupuncture, cauterization

Acupuncture in ancient China

Acupuncture may be defined as the insertion of needles into specific points on the body to produce a healing response. Each acupuncture point has specific actions when stimulated. Acupuncture is used all around the world, either along or in conjunction with Western medicine, to treat a wide variety of conditions in every species of animal. (International Veterinary Acupuncture Society)

Veterinarians in the United States of America (USA) may use acupuncture in their clinics (Lin and Panzer, 1994)

Classical Chinese acupuncture theory originally recognized 365 acupuncture points in people based on a "cosmological correspondence" between the number of points and the days of the year. (Ramey et al,2001)

The definition of acupuncture differ from past to recent time. in classical Chinese the term acupuncture (Zhen), conventionally translated as "needles" or "needling," actually encompasses the widest possible range of interventions, from acupuncture to therapeutic phlebotomy, surgery, and various forms of cauterization. The objects in question can be needles, but also lancets, elongated blades, and even metal branding irons.

And the earliest Chinese medical texts describe cauterization (i. e., moxibustion:), compresses, fumigation, medicinal baths, minor surgery, magical incantations, ritual movements, massage, cupping, steaming, pressure with stones, and some. (Imire et al, 2001)

Quan Ji Tong Xuan Lun (a dissertation on the treatment of sick horses), published during the Yuan dynasty (AD 1279 – 1368), described the treatment of sick horses by acupuncture and moxibustion. (Lin and Panizer, 1994), and author reported that: In 1608, one of the most significant writings on veterinary acupuncture, Yuan Heng Liao Ma Ji (Yuan and Heng's Therapeutic Treatise of Horses), was published by the Yu brothers, Yu Ben-Yuan and Yu Ben-Heng, two of the most famous veterinarians in Chinese history. (Ramey et al, 2001)

Chinese practiced the medicine of horse include other methods like herbal medicine, bloodletting.

Chinese herbal medicine is another main branch of traditional medicine in China which is capable of making a similar contribution to international veterinary practice. the veterinarian Zhao Fu performed blood-letting from the jugular vein of horses as a treatment for 'lung heat' during the reign of the Zhou Emperor Mu (947 – 928 BC). Another famous veterinarian, Sun Yang, alias Baile, wrote Baile Zhen Jing (Baile's Canon of Veterinary Acupuncture) at the time of Qin Mu-Gong (659 – 621 BC).

And the treatise covers many aspects of equine medicine and discusses the use of acupuncture, moxibustion and herbal medicine. In addition, the Yu brothers published two further treatises on the treatment of

oxen and camels. During the Qing dynasty (AD 1644 – 1911), Huo Shou Ci Zhou (Humane Care of Animals) was published in 1873, containing a large section on herbal medicine. (Lin and Panizer, 1994)

Horses in ancient Arab

The horse was very important for Arabs especially resident in desert (Sahara), because it was a suitable for fast transport, carrying and fighting against enemy who attack the trip, other sporting and social purposes as marriage as a bride price and, horses were resemble a highly economical values and the horse was a standard for economic state. (Mohamed,2011)

The Prophet Mohammed loved horse, and his and urged his friends to breed and rearing it.

And learned Arabian once wrote,"Paradise on earth is to be found on the back of a horse, in the pages of a book, and in the arms of the woman you love." (Bickel, 1965), The Mameluke Ahmed Ibn Tulun captures Egypt and builds a hippodrome for his best horses. (Davis, 2007)

The effect of many drugs has been appraised experimentally by trails in the horse, and what has been learned in that way many times has been applied to the human being. (Bickel, 1965),

Medicine of horse found in many old civilizations. In Arabic civilization it developed from pre Islam where recorded in Arabic poetry, as oral. and Arabs veterinarians in these era used oil, tar and cauterization in treatment of animals.

Veterinary medicine in ancient Arabs

Medicine of horse in ancient Arab depend on, clinical examination, include pulse, temperature respiration, physical examination of skin include brightness, elasticity etc., appearance, gait and physical examination feces, urine, and its relation with internal diseases, as digestive system, (digestive canal, liver, stomach, intestine). And consider a nutrient a environmental. The treatment depend on: herbal medicine, especially garlic as antibiotic cauterization, bloodletting, zoo therapy, hygienic measures, sporting, balance nutrient, same times poultices.

For example, the king of Yemen, AL Ashraf Omar Ibn YOSEf, Al Ghasany (596 AH), who wrote in his book in veterinary medicine, diagnosis and treatment animals (Equiue, bovive, ovive and camelidae), he reported a treatment of 75 diseased horse, where he used a herbal (H) medicine only in treatment of 18 one of them, H and blood letting in 7, H and cauterization in 4, H and cauterization and surgery in 3, H and surgery and zoo therapy in one, and surgery in 12, cauterization 7, blood letting in 6, zoo therapy in 2, zoo therapy, herbal and cauterization, surgery and cauterization in 5, cauterization and blood letting 1, herbal and poison of scorpion 1, oil 1, shooing 2.

Cauterization is one of the oldest means of treatment in the world: by glowing iron rods local 3rd degree burning marks are made in the skin. The site of applications varies for different diseases. Generally the location is anatomically related to the sites of the illness.

The Arab Medicine has influenced the European Medicine in the medieval times and cauterization became an important tool in the treatment of mankind and animals. (Selen, 2004)

Ancient and Arabic medicine

the Islamic pharmacopoeia contained two hundred new plants, a great many of which are still used at the present time, and it may be well here to mention a few of the most important.. (Ogden, 1926)

Herbs played a major part in Egyptian medicine. The plant medicines mentioned in the Ebers Papyrus for instance include many medical herbs. (Patrick et al, 2009). Ancient herbal medicine:

Garlic was an important healing agent then just as it still is to the modern Egyptian and to most of the peoples in the Mediterranean area and Minerals and animal products were used too. Honey and grease formed part of many wound treatments,. (Aboelsoud,2010)

Acupuncture between Arabs and Chinese

Arabic medic Medieval Arabic medicine also assumes a correlation between internal organs and at least one external point on the body in their tradition of cauterization, as seen in the Huinhui Yaofang (Muslim Medical Recipes), four chapters of which survive in a Ming dynasty copy. (Ramey et al, 2001)

In fact, the closest early parallel to the Bo Le diagram and the whole tradition associated with it can be found in the Arabic veterinary literature, namely in the veterinary manual of the Mamluk veterinarian Abu Bekr, a book dating from the first third of the 14th century. some special cauterization brands are given in the text against various ailments in addition to one special cut for use against muscle tears. (Buell et al, 2010)

From previous, it is clear that the ancient Arabs and Chinese used acupuncture as a method for treatment in parallel time, and nearly by same technique.

(1) (2) (3)

(4) (5) (6)

Fig. 1 Zootherapy in ancient Arabic manuscript (AAM), Manafea alhayawan, Ibn Bakhtisua; Fig. 2 Medical plant in, (AAM), mofradat aladwia, Ibn Al bytar; Fig. 3 Treatment of horse, in(AAM), albatra, Ibn Alahnaf; Fig. 4,5,6 Cauterization and branding of horse, Abu Beker 14th century, veterinarian in Egypt.

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Occurrence of Anti-*toxoplasma gondii* and *Neospora caninum* Antibodies in Camels (*Camelus dromedarius*) in Center of Iran

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Summary: During the period December 2008 to September 2009, we examined 254 serum samples (164 in hot-dry weather and 90 in cold-dry weather) from dromedary camels distributed all over the Yazd province, center of Iran. IgG antibodies were assayed by the modified agglutination test using whole tachyzoites of *Toxoplasma gondii* and *Neosporacaninum*, incorporating 2-mercaptoethanol, MAT and NAT, for *T. gondii* and *N. caninum*, respectively. Anti-*T. gondii* antibodies were found in 37 (14.56%) of 254 camels with titers of 1:20 in 7, 1:40 in 6, 1:80 in 8, 1:160 in one, 1:200 in 4, 1:400 in 6, 1:800 in 4, and 1:1600 in one camel. Ten of 254 (3.94%) camel sera had antibodies to *N. caninum* with titers of 1:20 in 6 and 1:40 in 4 camels. There was no difference between presence of antibodies for both parasites in male and female camels and in different weather conditions, but occurrence of anti-*T. gondii* antibodies was more in older camels.

Introduction

Toxoplasma gondii and *Neosporacaninum* are two closely related cyst forming apicomplexan protozoa that infect many of the warm-blooded animals [1,2]. They can cause abortion in ruminants [3 – 6]. *T. gondii* infection is prevalent in all of the warm-blooded vertebrates, and *N. caninum* is a major pathogen for cattle and dogs, which occasionally causes clinical infections in other animals including camels [1]. *T. gondii* is widely prevalent in the human beings and animals in Iran [7 – 13]. Dromedary camels (*Camelus dromedaries*) are important multipurpose animal of arid and semi-arid parts of the world. In Yazd province they are mainly kept for the purpose of meat production. They get slaughtered in registered industrial slaughter houses in Iran, under high hygiene controls and inspections. According to the last enumeration, there have been about 154000 camels in Iran, 21830 of them counted in the Yazd province [14]. The objective of the present study was to investigate the occurrence of anti-*T. gondii* and *N. caninum* antibodies in dromedary camels in Yazd province located in center of Iran.

Material and methods

From December 2008 to September 2009, five different regions were selected from 11 camel-rearing areas in Yazd province for two-stage cluster random sampling. Factors likely affecting seropositivity rate in analyses were age, gender and weather conditions. A total of 254 dromedary camels were included, 164 from hot-dry seasons and 90 from cold-dry seasons. The tested animal population comprised 207 males and 44 females. Blood samples were collected from camels of the ages ranging from 6 months to 30 years. Animals did not have any clear symptom of diseases at the time of sampling. Blood samples were centrifuged at 1000 × g and sera were stored at −20℃ until analysis.

The sera were tested for the presence of *T. gondii* antibodies using the MAT based on direct agglutination of fixed parasites with sera pre-treated with 2-mercaptoethanol to prevent non-specific IgM agglutination, as described before[15,16]. The sera were also tested by the *Neospora* agglutination test (NAT), asdescribed by Romand et al. [17]. This test is identical to MAT for *T. gondii*, but *N. caninum* tachyzoites are used instead of *T. gondii*.

The results obtained from serum evaluation, were analyzed statistically by logistic regression using SPSS software, version 16. Also Chi-square test was performed for *T. gondii*, and Fisher's exact test was used for analyzing *N. Caninum* results. Alpha was 0.05 for all tests.

Results and discussion

Anti-*Toxoplasma gondii* antibodies were found in 37 out of 254 camels (14.57%) with titers of 1:20 in 7, 1:40 in 6, 1:80 in 8, 1:160 in one, 1:200 in 4, 1:400 in 6, 1:800 in 4, and 1:1600 in one camel. *N. caninum* antibodies were detected in 10 out of 254 tested camels

giving 3.94% seropositivity rate with titers of 1:20 in 6 and 1:40 in 4. From 208 male camels, 31 (14.90%) and 8 (3.85%) were positive for *T. gondii* and *N. caninum antibodies*, respectively. From 46 females, 6 (13.04%) were positive for *T. gondii* and 2 (4.35%) for *N. caninum*. Analysis with logistic regression revealed that higher ages of camels were significantly associated with more *T. gondii* infection. Odds ratio (OR) was 1.216 with 95% confidence interval (CI) ($P < 0.001$). However no significant association was seen between age and occurrence of *N. caninum* (OR = 1.092, CI = 95%) ($P > 0.05$). The frequency of infection among both sexes and both weather conditions was similar for both parasites, and there was no relation between occurrence of *T. gondii* or *N. caninum* and those factors ($P > 0.05$). The results of this study are shown in Table 1.

Table 1 *T. gondii* and *N. caninum* specific antibodies in dromedary camel in Yazd, Iran according to gender and weather conditions

	n	*T. gondii* Seropositivity rate (#)%	*N. caninum* Seropositivity rate (#)%
Gender			
Male	208	(31)14.90	(8)3.85
Female	46	(6)13.04	(2)4.35
Weather condition			
Hot-dry	164	(24)14.63	(6)3.66
Cold-dry	90	(13)14.44	(4)4.44
Total	254	(37)14.57	(10)3.94

The 14.57% prevalence of *T. gondii* antibodies in the present study is similar to 17.4% prevalence of *T. gondii* foundin Egypt [18] and 16% in Saudi Arabia [19]. Hilali et al. [18] and Hosseininejad et al. [20] also showed a similar result for prevalence of *N. caninum* (3.72% and 3.22% respectively) in Egypt an Iran. However in a study by Wernery et al. [21] in UAE, 13.7% of examined camels found to be seropositive to *N. Caninum* by ELISA. Using IFAT, Sadrebazzaz et al. [22] showed that antibodies against *N. caninum* and *T. gondii* were present in 5.83% and 4.16% respectively of camels from North-East of Iran. This difference in results may be due to different procedures, the initial serum dilution or different climate and weather conditions. The data suggest that occurrence of toxoplasmosis in one-humped camels in center of Iran is relatively considerable, and consumption of camel meat may pose a risk to humans in the area of study. In contrast to the occurrence of clinical toxoplasmosis [23], clinical neosporosis in camels has not been yet reported. Toxoplasmosis is a globally distributed zoonosis with serious impact on unborn fetuses and also immunosuppressed individuals [24, 25]. *T. gondii* is responsible for approximately 21% of all deaths attributed to foodborne pathogens in the US, and the CDC estimates that 50% of all human exposures to *T. gondii* are foodborne [26]. In Europe, up to 63% of human *Toxoplasma* infections are attributable to the consumption of undercooked or cured meat products [27].

Conclusions

Although our results cannot provide an estimate of the percentage of infected camel meats, but consumption of camel meat may be one of the sources of infection for humans in center of Iran. Therefore, meat and other edible parts of animals should be cooked thoroughly before consumption.

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Welfare of Barred Plymouth Rock Poultry Flocks Supplemented with Zinc, Vitamin C and L-arginine during the Hot Summer Period using a Mathematical Assessment Model

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Summary: The mathematical animal welfare (AW) assessment model in poultry under different conditions has gained increasing importance.

The purpose of the present study was to evaluate the welfare of Barred Plymouth Rock breeder flocks whose feed was supplemented with either 1% L-arginine or zinc and vitamin C during the hot summer days, using a mathematical assessment model. The poultry welfare (PW) was scored on the basis of birds' behaviour, plasma corticosterone and several blood biochemical parameters, and microclimatic parameters. The poultry welfare score of control Barred Plymouth Rock flock was PW = 33.33%, that of the flock supplemented with L-arginine-PW = 66.67%, and the flock supplemented with zinc + vitamin C-PW = 73.33%.

Introduction

Very often, poultry reared in organic farming systems in moderate climatic regions suffer from heat stress resulting from the high summer ambient temperatures, which worsen their PW. In such instances, many researchers suggest dietary supplementation of suitable trace elements, vitamins or minerals to alleviate the stress. One option according to Wiesinger [1] is the dietary arginine intake, while another consists in adding antioxidant compound – Zn or vitamin C [2] and [3]. The reported results proved reduction of the negative effect of heat stress and improved PW.

The purpose of the present study was to provide an numerical evaluation of Barred Plymouth Rock welfare during the hot summer period, after dietary supplementation of either 1% L-arginine or the combination 35 mg/kg zinc and 250 mg/kg vitamin C (Zn + VIT C) using the changes in birds' behaviour, plasma corticosterone and some blood biochemical indices.

Material and methods

The experiments were performed with 87 Barred Plymouth Rock chickens at the age of 48 weeks from May 26 to July 26, 2011. The birds were reared in a organic farming system and divided in 3 groups. Each group (n = 29) was housed in identical sleeping pens (3.50/2.50/2.75 m) were equipped with perches and two-floor wooden nests. Each yard was 9.20/24 m with perennial broadleaf trees in the middle. Yards were provided with tubular feeders and with watering troughs ensuring feeding and drinking widths of 10 and 3 cm. The three groups were fed freely with the same compound feed according to birds' category. During the hot period (from June 25 to July 25), the diet of experimental groups was supplemented with either 1% L-arginine ("Roanal", Budapest, Hungary) or with 100 mg/kg Zinteral 35 (Lohmann animal health, Germany, containing 35 mg zinc/kg as zinc oxide) together with 250 mg/kg vitamin C (Zn + VIT C) (L-acidum ascorbicum, SHIJIAZHUANG Co. Ltd).

Microclimatic conditions were determined by routine methods. Blood samples for analysis were obtained from randomly selected six control birds during thermoneutral period (26th May), and during hot summer period (26th July) from each group from v. subcutanea ulnaris in vacutainers. The plasma corticosterone was assayed with an immunoenzymatic ELISA kit. Blood biochemical indices glucose, cholesterol, creatinine, total protein and triglycerides were determined on an automated biochemical analyzer.

The behaviour of parent flocks was recorded with a video camera for 12 hours during 4 consecutive days accounting the number of birds engaged in specific forms of behaviour: ingestive (ingestion of water or food), gregarious (moving, resting, egg-laying, dust bathing and feather cleaning), sexual and agonistic behaviour.

The PW assessment score was calculated by a modification of the system of Bozakova [4] based on the concept of animal welfare of the UK Farm Animal Welfare Council [5]

Statistical processing was performed by one-way ANOVA at significance level $P < 0.05$.

Results and discussion

During the summer, the average ambient temperature in the birds' living area was substantially

higher that the veterinary requirements for this category birds (Table 1).

Table 1 Microclimatic parameters during the hot period

Periods	Ambient temperature(℃)	Air humidity (%)	Air velocity (m/s)	NH_3 (ppm)	Light intensity (lx)
Thermoneutral period	19.44 ± 2.5	66.07 ± 1.59	0.50 ± 0.04	traces	65.00 ± 3.27
Hot summer period	31.24 ± 0.88	54.75 ± 1.25	0.41 ± 0.03	traces	87.90 ± 4.34
Reference values	18 – 25	50 – 70	0.2 – 0.5	< 15	30 – 60

It provoked a prolonged heat stress, manifested with marked behavioural changes-statistically significantly lower number of feeding, egg-laying, feather-cleaning, dust bathing and mating birds but increased number of drinking and resting control birds, compared to the thermoneutral period, (Table 2). Their blood corticosterone, glucose, cholesterol, creatinine and triglycerides (Table 3) were significantly higher compared to the thermoneutral period.

Similar changes in the behaviour and corticosterone levels of broiler chickens and layers under heat stress were reported by Ensminger [6]; Star [7] and Sahin [3]. On the basis of behavioural, corticosterone and biochemical changes, the five freedoms were scored (Table 4) and the total PW score was calculated using the method of Bozakova [4] – PW = 33.33%.

There were more feeding, egg-laying, resting, feather cleaning, dust bathing and mating experimental birds supplemented with 1% L-arginine, as well as lower walking and aggressive chickens compared to controls, (Table 2). Blood corticosterone, glucose, cholesterol, creatinine and triglycerides were lower than controls, (Table 3). The positive effects of the supplement were attributed to the inhibiting role of nitric oxide (a metabolite of arginine) on ACTH and corticosterone secretion [8].

The reduced negative impact of heat stress has resulted in higher scores of F_1, F_2, F_4, and F_5 freedoms vs controls (Table 4). Thus the total PW score in arginine-supplemented birds was PW = 66.67%.

Similar changes in the behaviour, corticosterone and biochemical, parameters were observed in birds receiving Zn + VIT C compared to controls, (Table 2; Table 3). There were significantly more cleaning and dust bathing chickens compared to the arginine group. Feather cleaning and dust bathing are reliable indicators of poultry welfare. The blood triglycerides in Zn-supplemented birds have further decreased compared to arginine-supplemented (Table 3). The lower plasma corticosterone is due to the antioxidant and antistress effect of Zn + VIT C combination. Being a co-factor of essential antioxidant enzymes Cu/Zn superoxide dismutase [9], zinc limits the excessive secretion of corticosterone. Vitamin C reduces corticosterone levels by including it in gluconeogenesis during stress [10]. This way, both supplements act synergically in the reduction of heat stress. On that basis the total poultry welfare score of the group supplemented with Zn + VIT C was PW = 73.33%.

Table 2 Number of Barred Plymouth Rock breeders, supplemented either with 1% L-arginine or with zinc and vitamin C exhibiting a specific type of behaviour (mean ± SEM, n = 29)

Behaviour	Thermoneutral period		Heat summer period					
	Control group	%	Control group	%	Arginine group	%	Zn + vitamin C group	%
Feeding	6.80 ± 0.63	23.45	4.91 ± 0.66$^{\hat{}}$	16.93	7.86 ± 0.58***	27.11	7.5 ± 0.62**	25.86
Drinking	4.34 ± 0.30	14.97	7.16 ± 0.38$^{\hat{}\hat{}\hat{}}$	24.69	7.16 ± 0.44	24.70	6.77 ± 0.45	23.34
Egg-laying	1.43 ± 0.23	4.93	0.61 ± 0.13$^{\hat{}\hat{}}$	2.12	1.09 ± 0.13*	3.76	0.96 ± 0.14*	3.31
Moving	8.09 ± 0.41	27.9	7.41 ± 0.23	25.55	3.00 ± 0.29***	10.34	2.84 ± 0.29***	9.79
Resting	2.30 ± 0.29	7.93	5.93 ± 0.56$^{\hat{}\hat{}\hat{}}$	20.45	4.52 ± 0.58*	15.59	3.84 ± 0.57**	13.24
Feather ceaning	0.86 ± 0.13	2.97	0.39 ± 0.10$^{\hat{}\hat{}}$	1.34	1.14 ± 0.21***	3.93	1.73 ± 0.18$^{***\#}$	5.97
Dust bathing	0.64 ± 0.12	2.21	0.09 ± 0.04$^{\hat{}\hat{}\hat{}}$	0.31	2.21 ± 0.36***	7.62	3.36 ± 0.44$^{***\#}$	11.59
Aggression	1.57 ± 0.13	5.41	1.23 ± 0.18	4.24	0.23 ± 0.07***	0.79	0.14 ± 0.05***	0.48
Sexual behaviour	1.91 ± 0.16	6.59	0.89 ± 0.11$^{\hat{}\hat{}\hat{}}$	3.07	1.66 ± 0.17***	5.72	1.73 ± 0.17***	5.97

$^{\hat{}}P < 0.05$, $^{\hat{}\hat{}}P < 0.01$, $^{\hat{}\hat{}\hat{}}P < 0.001$: statistically significant behaviour difference between thermoneutral and heat summer period in control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$: statistically significant difference between control and experimental groups during hot summer period; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$: statistically significant difference between Arginine supplemented and zinc and vitamin C supplemented groups during hot summer period.

Table 3 Blood corticosterone levels and biochemical indices in breeders supplemented either with 1% L-arginine or with zinc and vitamin C (mean ± SEM, n = 6)

Parameters	Thermoneutral period	Hot period		
	Control group	Control group	Arginine group	Zn + vitamin C group
Corticosterone, nmol/L	79.23 ± 4.71	177.25 ± 4.35$^{\hat{}\hat{}\hat{}}$	99.53 ± 3.62***	94.18 ± 6.08***
Glucose, mmol/L	6.47 ± 0.39	10.79 ± 0.27$^{\hat{}\hat{}\hat{}}$	7.63 ± 0.32***	7.30 ± 0.20***
Total cholesterol, mmol/L	2.61 ± 0.12	3.74 ± 0.20$^{\hat{}}$	2.93 ± 0.07**	2.97 ± 0.08**
Total protein, g/L	104.78 ± 3.52	52.98 ± 4.82$^{\hat{}\hat{}\hat{}}$	78.16 ± 5.34**	89.02 ± 3.63***
Creatinine, mol/L	55.59 ± 5.38	111.59 ± 3.33$^{\hat{}\hat{}\hat{}}$	82.39 ± 4.33***	78.92 ± 2.77***
Triglycerides, mmol/L	4.42 ± 0.42	10.35 ± 0.40$^{\hat{}\hat{}\hat{}}$	8.34 ± 0.54**	6.42 ± 0.48$^{***\#}$

$^{\hat{}}P < 0.05$, $^{\hat{}\hat{}}P < 0.01$, $^{\hat{}\hat{}\hat{}}P < 0.001$: statistically significant difference in control group between thermoneutral and heat summer period; $^{*}P < 0.05$, $^{**}P < 0.01$: statistically significant difference between control and experimental groups during hot summer period; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$: statistically significant difference between Arginine supplemented and zinc and vitamin C supplemented groups during hot summer period.

Conclusions

The poultry welfare score of breeder flocks suffering from heat stress was PW = 33.33%.

The 1% L-arginine supplemented birds were characterized with positive behavioural changes and reduced corticosterone and some biochemical indices compared to controls, as well as an increased welfare score to PW = 66.67%.

The supplementation with 35 mg/kg zinc and 250 mg/kg vitamin C was reflected positively on behaviour, blood corticosterone and some biochemical indices in the breeders. Their welfare score increased to PW = 73.33% due to the synergic heat stress reducing effect of both compounds.

Table 4 Welfare assessment scores of Barred Plymouth Rock breeders supplemented either with 1% L-arginine or with zinc and vitamin C during the hot summer period

Poultry welfare assessment		Free range system		
Freedom	Degree	Control group	Arginine group	Zn + vitamin C group
Freedom from thirst and hunger-F_1	0-excessive thirst and hunger	1	2	2
	1-limited thirst and hunger			
	2-lack of thirst and hunger			
	3-excessive feeding and drinking			
Freedom from discomfort-F_2	0-excessive discomfort	1	2	2
	1-limited discomfort			
	2-limited comfort			
	3-full comfort			
Freedom from pain. injury disease-F_3	0-exhausting disease	2	2	2
	1-limited disease			
	2-occasional pain and injury			
	3-lack of pain and injury			
Freedom to express normal behaviour-F_4	0-behaviour disturbance	1	2	3
	1-limited behaviour expression			
	2-moderate expression			
	3-full expression			
Freedom from fear and distress-F_5	0-fear and distress	0	2	2
	1-limited fear and distress			
	2-partial freedom			
	3-full freedom			
Total score		5	10	11
Poultry welfare assessment, %		33.33	66.67	73.33

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Water Quality and Blood Biochemical Parameters as Indicators for Welfare Assessment in a Sturgeon Private Farm

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Summary: Lately, *Acipenseriformes* are considered to be in danger due to over fishing, dams building and to life environment degradation, which is why the focus is on the development of sturgeon farms by accessing European funds.

This study aims to assess the sturgeons' welfare in such a farm from Calarasi County.

In order to assess the water quality there were taken samples from several checkpoints in farm and there were determined by using Nova 60 photocolorimeter the following physical and chemical parameters: temperature, pH, turbidity, dissolved oxygen, chlorine, nitrates, nitrites, phosphorus, iron, copper, ammonia, sulfates, phenols and detergents.

There were also determined the water microbiological parameters, respectively total coliforms and fecal ones.

For biochemical examination there were taken blood samples from several sturgeon specimens and were analyzed by using dry biochemistry technique (Vettest 8008 analyzer) the following: blood urea nitrogen, creatinine, uric acid, calcium, total proteins, albumin, globulins, alanine aminotransferase, aspartate aminotransferase, gamma glutamyl transferase, triglycerides, carbohydrates, lactate dehydrogenase, phosphorus, magnesium, alkaline phosphatase, total bilirubin, cholesterol, ammonia, amylase, lipase and creatine kinase. The results interpretation was made according to the reference values for sturgeon.

Following the researches, it was found that most physical and chemical parameters of water were within normal limits accepted for sturgeon, except nitrates and sulphates. Water microbiological parameters exceeded the limits in all samples.

Regarding blood serum biochemical parameters, the obtained values were normal, except alanine aminotransferase, aspartate aminotransferase, triglycerides and alkaline phosphatase.

Linking the water quality parameters values with those for blood biochemical parameters, the welfare of sturgeons in the farm may be rated as average.

Introduction

Sturgeons are valuable fish, known for their nutritional qualities and taste of both meat and roe, as well as for their size compared to other species, and for being among the most ancient fish on earth. In our country, there is evidence that on the tables of the Geto-Dacian state founders could be found sturgeon steaks and caviar.

Sturgeon meat is easily digestible, with great nutritional value, considering the high content of amino acids, vitamins A, B and D, phosphorus, nonessential fatty acids.

Now, due to over fishing, water pollution and dams building, *Acipenseriformes* are in danger, which is why the focus is on the development of sturgeon farms by accessing European funds in order to breeding, raising and restocking natural basins in our country.

Researches in the present paper were conducted in such a fish farm from Calarasi County.

Material and methods

Sturgeon welfare assessment from Tamadau farm was made considering two indicators, namely: the quality of fish living environment and blood biochemical parameters.

In order to assess water quality, samples were collected of which have been determined physical-chemical parameters (temperature, turbidity, pH, dissolved oxygen, nitrates, nitrites, phosphorus, residual chlorine, iron, copper, ammonia, sulfates, phenols, detergents), and microbiological parameters (total coliforms and fecal coliforms).

Blood samples were taken from sturgeons by caudal vein puncture with ventral election point and there have been determined 22 serum biochemical parameters: blood urea nitrogen (BUN), creatinine, uric acid, calcium, total proteins, albumin, globulins, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), triglycerides, carbohydrates, lactate dehydrogenase (LDH), phosphorus, magnesium, alkaline phosphatase (ALP), total bilirubin, cholesterol, ammonia (NH_3), amylase, lipase and creatine kinase (CK).

Water quality parameters were determined by using NOVA 60 photocolorimeter and blood biochemical

parameters by Vettest 8008 dry biochemistry analyzer.

Results interpretation was made according to the reference values provided for sturgeon [3,4].

Results and discussion

Average values of physical and chemical parameters of water are presented in Table 1.

Table 1 Average values of water physical and chemical parameters

Determined parameters	Sampling points					Admitted limits[3,4,6]
	Water source	Stilling basin	Water inlet	Pond center	Water outlet	
Dissolved oxygen (mg/l)	7.0	7.0	8.0	7.5	7.2	>6.0
Residual chlorine (mg/l)	0.14	0.14	0.14	0.09	0.12	<0.3
pH	7.7	7.7	7.7	7.6	7.4	7 – 8
Nitrates (mg/l)	8.0	8.0	8.0	12.0	13.0	10.0
Nitrites (mg/l)	0.063	0.063	0.063	0.078	0.093	<0.1
Phosphorus (mg/l)	0.8	0.8	0.8	1.4	0.9	2.0
Iron (mg/l)	0.07	0.07	0.07	0.07	0.07	1
Copper (mg/l)	0.09	0.09	0.06	0.05	0.05	<0.3
Ammonia (mg/l)	not detected	not detected	not detected	not detected	not detected	<0.05
Sulphates (mg/l)	20.0	20.0	20.0	25.0	20.0	2 – 7
Phenols (mg/l)	0.10	0.10	0.10	0.20	0.19	1 – 2
Detergents (mg/l)	0.05	0.05	0.05	0.05	0.04	<0.1

Analyzing data from the table, it is found that dissolved oxygen ranges both within the limits stipulated by Order 161/2006 [6] and into the reference values for sturgeon [3, 4].

Dissolved oxygen content in the fish living environment is very important for sturgeon life because only in clean water it tends toward the saturation value depending on temperature and atmospheric pressure. Excess fish food may decrease dissolved oxygen content due to biological and biochemical processes in the water.

Residual chlorine and water pH are within the reference values for sturgeon.

Water reaction is neutral. Any change in water pH may adversely affect the health of fish: water acidic reaction can lead to respiratory changes, lack of interest in feed, reduced mobility, gasping at the surface, slowing growth and, ultimately, sturgeon's death [2].

Nitrites, phosphorus, iron, copper, ammonia, phenols and detergents recorded values falling within the reference standards for sturgeon.

Deviations from the reference values were recorded for nitrates, the results exceed the limit approximately 2 times, which is not a threat to fish health.

For sulfates, there was recorded significant exceeding, causing nervous disorders and skin lesions in fish [1].

In Table 2 are shown water microbiological parameters values, namely total and fecal coliforms in water samples taken from three points.

Table 2 Average values of water microbiological parameters

Sampling points	Determined parameters	
	Total coliforms (No./ml water)	Fecal coliforms (No./ml water)
Water inlet	620	428
Pond center	905	920
Water outlet	800	700
Admitted limits [5]	500	100

Analyzing the results, it is found that the values obtained are above the admitted limits in all sampling points. This is explained by the high microbiological load of the water source that supplies the sturgeon farm.

Blood biochemical parameters values are shown in Table 3.

Analyzing the results, it is found that for the most of the determined biochemical parameters (BUN, uric acid, calcium, total protein, LDH, phosphorus, total bilirubin, cholesterol, ammonia, creatine kinase, amylase, lipase, GGT) values obtained are within the standard limits for *Acipenserids*.

The changes of the determined values are represented by significant decrease of triglycerides and slight decrease of magnesium and globulins, related to the retention stress during blood sampling, and by increased alkaline phosphatase generated by adaptation stress, given that fish had been recently transferred from one pond to another and had had feeding problems [7].

There were also noticed a slight decrease of ALT and an increase of AST activity, most likely related with fish muscular injuries caused by blood sampling.

Table 3 Values of blood serum biochemical parameters

Determined parameters	Obtained values		Reference values Female/male
	Female	Male	
BUN (mg/dl)	4.0	3.0	3.69 ± 0.64/3.78 ± 0.84
Creatinine (mg/dl)	0.0	0.0	0.344 ± 0.048/0.34 ± 0.06
Uric acid (mg/dl)	< 0.1	< 0.1	0.02 ± 0.003/0.03 ± 0.005
Calcium (mg/dl)	5.8	6.9	8.52 ± 2.76
Total proteins (g/dl)	3.5	4.2	4.51 ± 1.0/5.5 ± 0.94
Albumin (g/dl)	1.0	1.0	1.26 ± 0.29
Globulins (g/dl)	2.8	2.9	3.63 ± 0.84/4.5 ± 0.69
ALT (U/l)	100.0	92.0	100.65 ± 1.18
AST (U/l)	220.0	459.0	265.6 ± 56.55
GGT (U/l)	0.0	0.0	0.02 ± 0.0035
Triglycerides (mg/dl)	250.0	270.0	699.6 ± 22.94
Carbohydrates (mg/dl)	62.0	52.0	61.62 ± 15.13/120.54 ± 26.74
LDH (U/l)	2300	2800.0	2007.15 ± 521.97
Phosphorus (mg/dl)	8.0	11.4	12.39 ± 0.267/9.009 ± 2.07
Magnesium (mg/dl)	1.81	2.27	2.79 ± 0.63/3.67 ± 0.85
ALP (U/l)	59.6	360	69.05 ± 13.04
Total bilirubin (mg/dl)	0.5	< 0.1	0.616 ± 0.0234
Cholesterol (mg/dl)	70.0	91.0	90 ± 40
NH_3 (mmol/l)	449	457	300 ± 170
Amylase (U/l)	0.0	0.0	0.001 ± 0.0003
Lipase (U/l)	0.0	0.0	0.004 ± 0.001
CK (U/l)	411	> 2000	2700 ± 1150

Conclusions

1. Physical and chemical parameters of water quality in the studied sturgeon farm have ranged, in most cases, within the reference values, except nitrates and sulfates.

2. Microbiological parameters of water recorded overvalues due to pollution of the farm water supply source.

3. Most of the blood serum biochemical parameters determined recorded appropriate values for sturgeon, except for the high alkaline phosphatase and the decrease of triglycerides. The increase of AST activity is most likely related with fish muscular injuries during blood sampling.

4. Correlating the results for the physical chemical parameters and microbiological parameters with the ones for the blood biochemical parameters, it results that the welfare of the sturgeons in this farm is rated as average.

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Dairy Cows' Welfare Assessment in a Private Farm from South of Romania

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Summary: Animal welfare has lately aroused interest of a growing number of consumers who become aware of its implications on public health, food safety, environmental protection and biological diversity.

In a private farm from southern Romania was assessed the welfare level of dairy cows based on microclimate factors (temperature, relative humidity, draughts, noise, light intensity), on blood biochemical indicators (albumin, globulins, total proteins, glucose, calcium, magnesium, blood urea nitrogen, alkaline phosphatase, phosphorus, aspartate aminotransferase, gamma glutamyl transferase, creatine kinase), and on animal health determined from consultation records and by individual clinical examination.

Microclimate parameters were measured with thermo-hygrometer, sound level meter, light meter, catathermometer and the biochemical blood indicators by using dry chemistry analyzer Vettest 8008.

The research found that the majority of microclimate parameters have appropriate values in relation with welfare standards, except draughts velocity, for which values slightly exceeding the standards.

Regarding the results of blood biochemical parameters investigation, for 80% of the samples the values were in reference interval and for the other 20% were recorded elevations for globulins and total proteins and decreases for blood urea nitrogen and glucose.

Of all animals which were clinically examined, 20% presents lameness due to the failure to comply with floor hygiene requirements.

Correlating microclimate factors with biochemical blood parameters and health of dairy cows, the welfare of animals in the farm can be rated as average.

Introduction

Lately, animal welfare raised the interest of more and more consumers, who have become aware of welfare implications on public health, food safety, environment protection and biological diversity [2].

Improving animal welfare is, on the one hand, a moral and ethical duty of man, on the other hand, a vital necessity for the prosperity and his existence on earth [4].

Semi-intensive system of rearing dairy cows combines specific elements of extensive rearing with elements of the industrial-intensive technology in order to increase the economic profitability of the farm [3].

Present researches aimed to improve knowledge regarding assessment of dairy cows' welfare by identifying microclimate factors and blood serum biochemical parameters with major influence, which would enable farmers to find the problems and to correct them.

Material and methods

In a private farm of dairy cows from the south of the country was assessed the livestock welfare level based on the microclimate factors, on blood serum biochemical indicators and on animals' health status.

There have been determined the following microclimate factors: temperature, relative humidity, air draughts velocity, noise and light intensities.

Also, blood samples were collected in two stages separated by three months interval, from a total of 10 dairy cows, with different ages and health status.

Of the blood samples there have been determined the following serum biochemical parameters: albumin (ALB), globulin (GLOB), total protein (TP), glucose (GLU), calcium (CA), magnesium (MG), blood urea nitrogen (BUN), alkaline phosphatase (ALKP), anorganic phosphate (PHOS), aspartate transaminase (AST), gamma-glutamyl transferase (GGT), and creatine kinase (CK).

Health problems were found by examining the records of consultation and treatments and by conducting detailed clinical examination of each cow.

Microclimate factors were determined by using the electronic thermo-hygrometer, sound level meter, light meter and Hill catathermometer, and blood biochemical parameters by using Vettest 8008 dry biochemistry analyzer.

Results interpretation was made for the microclimate factors in accordance with hygiene and welfare regulations and, for the biochemical parameters, values obtained were compared to the reference values for dairy cows.

Results and discussion

The values of the microclimate factors determined are given in Table 1.

Table 1 The average values of physical microclimate factors

Measurement place	Temperature (℃)	Relative humidity (%)	Noise intensity (dB)	Light intensity (lx)	Draughts velocity (m/s)
Shelter 1	17	65	56	62	0.77
Shelter 2	18	70	53	60	0.82
Shelter 3	17.5	72	49	47	0.79
Shelter 4	17	68	58	52	0.75
Optimal value	14 – 20	60 – 75	< 50 – 60	50 – 60	0.1 – 0.5

Analyzing data from the table it is found that the values determined for most parameters are optimal, except for the air draughts velocity, which are slight higher than those allowed by welfare standards.

The increasing of milk production and body weight, the improving of the reproductive indices (fertility, fecundity percentages) are influenced by temperature. High temperature in the shelter leads to a decreased feed consumption by 30% – 50% and a reduced milk production [1].

A photoperiod of 16 hours light increases milk production by 6%.

Average values of blood serum biochemical parameters are shown in Tables 2 and 3.

Table 2 Blood serum biochemical parameters in cows – A

Determined parameter/ measure unit	Animal identification number										Reference values
	6785		6782		1156		4816		85112		
	1*	2**	1	2	1	2	1	2	1	2	
BUN (mg/dl)	4	11.0	9	7	6	7	7	13	13	6	10 – 25
PHOS (mg/dl)	8.3	6.9	7.3	7.3	6.5	5.8	6.9	5.9	8.2	5.7	4.0 – 8.6
CA (mg/dl)	9.2	9.5	9.8	8.9	9.2	8.3	9.6	8.7	8.8	8.9	8.0 – 12.0
MG (mg/dl)	2.08	2.56	1.92	2.30	1.69	2.37	2.08	2.44	2.87	1.97	1.8 – 3.0
TP (g/dl)	6.1	7.2	6.6	7.3	7.7	6.7	7.1	8.0	8.0	8.3	6.2 – 8.0
ALB (g/dl)	2.5	2.4	2.6	2.6	2.5	2.2	2.5	2.9	3.0	2.4	2.5 – 3.5
GLOB (g/dl)	3.7	4.7	4.1	4.6	5.2	4.5	46	5.1	5.0	5.9	3.0 – 4.9
AST (U/l)	91	69.0	79	106	106	99	100	108	103	87	50 – 150
ALKP (U/l)	48	41	54	71	78	91	72	83	49	38	28 – 233
GGT (U/l)	20	31	48	29	43	44	34	51	36	44	0 – 87
GLU (mg/dl)	77	48	68	37	49	51	73	53	74	31	56 – 88
CK (U/l)	78	65	61	69	63	82	106	106	95	114	50 – 250

* first sampling stage; ** second sampling stage. The same as follows.

Table 3 Blood serum biochemical parameters in cows – B

Determined parameter/ measure unit	Animal identification number										Reference values
	6781		2954		9559		3990		3956		
	1*	2**	1	2	1	2	1	2	1	2	
BUN (mg/dl)	13	10	11	7	4	13	14	15	14	11	10 – 25
PHOS (mg/dl)	8.2	7.0	5.6	8.2	6.4	6.8	7.0	6.4	6.6	5.7	4.0 – 8.6
CA (mg/dl)	9.0	9.4	8.5	8.6	9.4	9.4	9.0	9.0	7.0	9.2	8.0 – 12.0
MG (mg/dl)	2.89	2.31	2.8	2.32	2.20	2.53	2.4	2.87	1.66	2.64	1.8 – 3.0
TP (g/dl)	8.1	7.5	6.6	7.2	8.9	9.2	7.5	9.0	6.8	8.6	6.2 – 8.0
ALB (g/dl)	3.0	2.8	2.5	2.5	2.8	3.4	2.9	3.2	2.7	2.9	2.5 – 3.5
GLOB (g/dl)	5.1	4.7	4.1	4.7	6.1	5.8	4.6	5.8	4.1	5.7	3.0 – 4.9
AST (U/l)	109	88	118	99	108	120	155	111	156	154	50 – 150
ALKP (U/l)	46	72	49	45	46	40	65	49	50	61	28 – 233
GGT (U/l)	35	41	34	51	57	35	34	42	47	47	0 – 87
GLU (mg/dl)	75	43	86	43	65	53	90	48	64	56	56 – 88
CK (U/l)	94	91	106	101	134	85	241	243	402	389	50 – 250

Analyzing the results in the table, it is found that in most samples, blood biochemical parameters had normal values, except for BUN and GLU where there were lower levels, and for GLOB and TP where values were higher.

Hyperglobulinemia is caused by chronic inflammatory diseases such as liver abscess, traumatic reticuloperitonitis and chronic pneumonia.

Low levels of BUN reflect the liver's ability to metabolize ammonia from urea, and hypoglycemia is due to ketosis.

Regarding the health of dairy cows, after examining the records of consultations and treatments and detailed individual clinical examinations was found a number of diseases such as mammitis, metritis, bronchopneumonia and a 20% prevalence of laminitis which are due to poor quality of the floor and to the defective hoof trimming made by sloppy stockmen.

Conclusions

1. Most of the microclimate parameters recorded normal values, except for the air draughts which had values slight higher than the standards set for dairy cows.

2. Blood serum biochemical parameters had values above the reference for 20% of the samples taken for GLOB and TP, and below these limits for BUN and GLU.

3. Health assessment of dairy cows studied revealed that 20% of the animals showed lameness.

4. Microclimate factors correlation with blood biochemical parameters values and animal health status has led to a rating of average welfare in the private farm in southern Romania.

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Behaviour of Buffalo Cows in the Milking Parlour: Entrance Order and Side Preference

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Summary: The aim of this study was to investigate dairy buffalo entrance order and side preference in the milking parlour. Two milking groups of buffalo cows were used. In both groups there was strong consistency of entrance order into the milking parlour. There was also evidence for high preference for the left or right side by individual animals. Twelve animals showed stall preference. Our results showed that buffaloes can present marked entrance order consistency and side preference. We concluded that buffaloes should be allowed to satisfy these preferences during the milking process.

Introduction

Detailed knowledge of farm animal behavioural characteristics enables the development of husbandry such that it can meet animal needs as much as possible. This can benefit the animals' welfare by allowing the animals to realise their full individual potentials, therefore maximising individual and overall herd productivity [12, 7]. Voluntary movements of cattle have been studied in different situations. Several studies have shown that a consistent order of entry into the milking parlour is a prominent feature of the social system of the dairy cattle [9, 4, 1]. In addition, it has been demonstrated that some cows are consistent in the choice of one side of the milking parlour, showing a clear side preference [5, 7, 4].

Milking routine can affect cows' welfare and some authors have suggested that drift from preferred behaviour may cause stress during milking [5, 4]. Buffaloes are more sensitive to stress stimuli then cattle. If animals are stressed adrenaline is secreted, and this may reduce the supply of oxytocin, necessary for milking [6]. This is exacerbated in buffaloes, which are difficult to milk, and milk ejection can easily be disturbed, and consequently stock-people commonly use exogenous oxytocin in order to allow a complete evacuation of milk from the udder [10, 6].

Studies on the behaviour at milking of dairy animals have mainly focused on cattle. While some research on milking order has been made on small ruminants [12, 11] little is known about dairy buffaloes. The aim of this study was to assess the consistency of the entrance order into the milking parlour and side preference of dairy buffaloes.

Material and methods

The study was carried on commercial dairy buffalo farms located in the Campania Region of Southern Italy, situated in the Volturno Plain (41°18′N, 14°15′E).

Animals were group-housed in a loose open-sided barn, with a concrete floor in the lying area bedded with dried manure solids. Two milking groups were used. In the first group, data from 120 milkings (from mid-March to mid-May 2012) from 55 buffalo cows (3 primiparous, 16 secondiparous and 36 multiparous) were collected, while in the second group 90 milkings (from the end of March to the mid of May 2012) of 36 buffalo cows (12 primiparous, 10 secondiparous and 14 multiparous) were considered. Buffaloes were milked twice a day with 2 × 6 auto-tandem milking parlour. The mean days in milk at the beginning of the experiment were 59 ± 27 d and 136 ± 88 d for groups 1 and 2, respectively. Milk yields per milking session at the beginning of the experiment were 6.2 ± 2.1 and 3.4 ± 1.1 kg for groups 1 and 2, respectively.

Before milking each group entered the waiting area (size of approximately 120 m^2) and the animals were free to choose their position in relation to entrance into the milking parlour, with no intervention by the stockman. The two sides of the parlour were identical to each other, and access to both sides was through pneumatically operated gates. In the parlour, cows stood nose to tail in

individual stalls with entry and exit through side passages. In each stall, the identity of the cow was automatically recorded through a pedometer fitted at the metatarsus. The milking equipment in the Farm was the ALPRO® system (De Laval, Sweden). This system allows the collection of all data from the milking parlour, including the milking position of each cow, milk yield, and time and duration of milking.

For each milking group, the consistency of entrance order was computed using the Kendall concordance coefficient. The correlations between mean entrance order vs. mean milk yield, mean days in milk and mean duration of milking were calculated using the Spearman correlation coefficient. For each cow, side preference in the milking parlour was assessed using the χ^2 one-sample test, with 50% as expected frequency. However, it was considered a side preference only if an animal chose a particular side for more than 80% of the milking sessions. For the animals showing side preference, the preference for one stall out of the six available in one side was assessed using the χ^2 one-sample test, with 16.7% as expected frequencies. It was considered stall preference only if an animal chose a particular stall on more than 34% of the milkings on the preferred side.

Results and discusson

Group 1

A strong consistency of entrance order into milking parlour was found ($W = 0.779$; $\chi^2 = 5046.81$; $P < 0.001$). Animals with higher days in milk tended to enter the milking parlour last ($r_s = 0.243$; $P < 0.10$). A negative correlation was found between duration of milking and order of entry in the milking parlour ($r_s = -0.265$; $P < 0.05$;).

All the animals showed a side preference. Twenty-eight cows (50.9%) preferred the right side of the milking parlour and the remaining 27 (49.1%) the left side. Forty-seven out of 55 animals were milked on 98% – 100% occasions on the preferred side, and the remaining eight on 87% – 97% occasions. Seven of the animals which showed a side preference also exhibited a stall preference.

Group 2

As for the other milking group, strong consistency of entrance order into milking parlour was also found ($W = 0.624$; $\chi^2 = 2030.48$; $P < 0.001$). Animals with higher days in milk entered the milking parlour last ($r_s = 0.334$; $P < 0.05$). No significant correlations were found among the other variables collected.

Thirteen cows (36.1%) preferred the right side of the milking parlour, 21 the left side (58.3%) and the remaining 2 (5.6%) showed no preference. Thirty out of 34 animals showing preference were milked 98% – 100% of the time on the preferred side, and the remaining four 85% – 96% of the time. Five of the animals showing a side preference also exhibited a stall preference.

Strong consistency of the entering at milking parlour in both groups shows that buffaloes do not enter the milking parlour randomly. This is similar to the results reported on cattle, sheep and goats [4, 12, 11, 3]. Taking in to account previous works on dairy cattle [4, 8], buffaloes seem to have higher consistency of entrance order to the milking parlour. Some authors have reported that higher yielding dairy cows [9, 4, 1] and goats [3] enter the milking parlour earlier. The current results with buffaloes are in agreement with those studies, which further found that older cows tend to enter milking parlour later, and it was suggested that this could be related to hierarchic rank in the group.

In the first group the side preference was very high, all cows preferred either the left (49.1%) or right (50.9%). In the second group preferences were: left 58.3% and right 36.1% and only 5.6% showed no preference. Paranhos da Costa & Broom (2001) found similar results with dairy cows but others [4, 5] also found side preference in dairy cows, but with lower frequencies. Factors which could affect side choice could be milking technique, social behaviour, neurological development, and daily routine, including interaction with humans [7, 4, 5]. Dairy cows tend to select the most attractive side or avoid the unattractive [5]; therefore each animal should be able to choose its favourite side of the milking parlour to feel comfortable during milking [4]. Entering the milking parlour from the non-preferred side, might result in stress response during milking [5], which would reduce the animal's milk yield [12]. Buffaloes are sensitive to changes in the environment and when they are uncomfortable with the situation they may withhold the milk [2]. Some buffalo cows in this study also showed stall preference, which could further indicate that they are very consistent in their milking routine, so it is important that buffaloes can choose freely the side and place in the milking parlour.

Conclusions

Buffaloes showed strong consistency in their entrance order into the milking parlour. Only two out of 91 animals showed no side preference. There was no overall preference for either the right or left side. Most animals showing preference were milked from 98% – 100% occasions on the preferred side. Twelve of the animals showing a side preference also exhibited a stall preference. These results indicated that buffalo can show marked entrance order consistency and parlour side preference.

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Increasing Milk Yield using a Cow Comfort Assessment System with Variable Weight for Parameters Depending on Their Score

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Summary: A scoring system for dairy cow comfort in free stall barns is developed that results in a single score for each farm. It consists of animal based parameters as well as environmental aspects, having a variable weight for all parameters, depending on their score. This system has been tested on dairy farms in the Netherlands, Mexico and Greece with a positive correlation between the scores and milk yield. Furthermore, there was a correlation of 0.84 with the Welfare Quality® Assessment Protocol for cattle in the Greek farms.

Introduction

Cow comfort receives substantial attention in modern dairy farming. Up to date, there is no report about the relation between the general level of cow comfort and milk yield. There have been studies with different approaches, for instance: animal behaviour, physiology, anatomy, health and immunity (McGlone, 2001), or based on facilities and environment (Barnett, 2007). Emphasis has been made on production related parameters such as lameness, water consumption and nutrition (von Keyserlingk et al., 2009). The health status is a major concern, it can be influenced by the cow-comfort level, but it is also of key importance for the well-being of a cow (von Keyserlingk et al., 2009). Milk yield is objectively measurable in an easy way. Cow comfort, however, is not as easy to assess if one wants an overall score (Fraser, 2003). In the design of a scoring system for cow comfort, several approaches can be chosen. One can look at the cows individually or as a herd, at one moment or over a certain time period, and one can include the environment as well. Furthermore, the time needed for the assessment should not be too long, in order to be applied as a tool in herd management programs. In this paper, a cow comfort monitoring system is presented that provides an overall score for cow comfort and its relationship with milk yield is determined.

Material and methods

The scoring system that has been developed is listed and explained in another paper (van Eerdenburg et al., 2013). Farms were assessed by trained investigators in three countries: The Netherlands (48), Mexico (55) and Greece (36). In the analysis, the level of milk production was correlated with the total score and with each chapter (Pearson correlation in SPSS 16.0). Because of the different climatic conditions in Mexico, Greece and the Netherlands, the data from each country were treated separately.

Comparison with Welfare Quality Assessment

In order to compare the newly developed system with the Welfare Quality Assessment, the final results of both scoring systems were correlated after conversion to a scale from 1 to 4: 1) Total score < 150; 2) 150 – 200; 3) 250 – 350; 4) > 350.

Results

The Dutch farms had a correlation of 0.34 ($P < 0.02$) between the number of points scored and the 305 day milk yield. The combined health factors were positively correlated with the combined other scored items on the Dutch and Greek farms ($r = 0.70$; $P < 0.001$, $r = 0.72$; $P < 0.001$). On the Mexican farms these were not correlated. Several chapters in the scoring system did have a significant correlation with the milk yield level. Examples are presented in Figures 1 and 2.

The execution of both systems differed substantially. The cow comfort score system concentrated particularly on the environment of the cow, and the Welfare Quality® protocol examined the condition of many individual cows. This meant that there was a substantial difference in time needed to examine the farms. For instance, by the cow

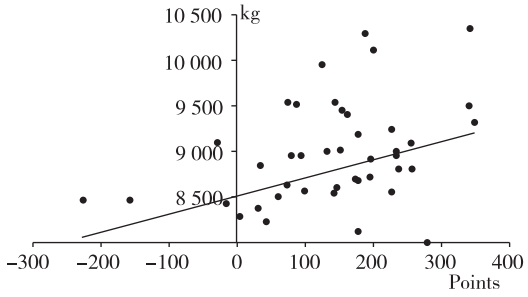

Fig. 1 Results of 48 farms in the Netherlands. The milk yield (305 day rolling herd average) was correlated with the cow comfort score ($r = 0.34$; $P < 0.02$)

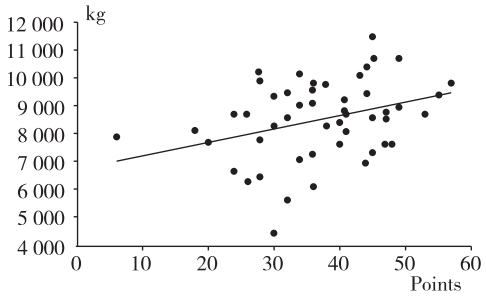

Fig. 2 Correlation between free stall comfort and milk yield in the Mexican farms ($r = 0.33$; $P < 0.02$)

comfort score system more cows are watched at the same time by which an average score is recorded, like cleanliness of the cows. With the Welfare Quality® protocol a substantial percentage of the cows is checked individually, the cleanliness of the lower hind legs, hind quarters and udder is noted by which an average is calculated. But as both systems were used more often, the execution went faster. Nevertheless, time needed for examining differed enormously. On a farm with 100 cows, applying the cow comfort score system lasted an hour and a half, applying the Welfare Quality® protocol took almost 7 hours. The results of both systems had a correlation of 0.84 ($P < 0.01$). Scores achieved by the individual farms using the cow comfort score system varied from 55 to 330 points. The overall assessment of the farms with the welfare quality protocol varied from not classified to enhanced. Standing idle was also correlated with the total score of the Welfare Quality protocol ($r = 0.87$; $P < 0.01$). The item standing idle had a correlation with the total score of the scoring system as well. In the Mexican farms it was 0.43 ($P < 0.01$), for the Dutch farms $r = 0.39$ ($P < 0.01$) and for the Greek farms 0.42 ($P < 0.01$). Without the health related items the correlations were 0.57, 0.50 and 0.53 ($P < 0.001$) respectively.

Discussion

The fact that the Mexican and Greek farms scored higher than the Dutch farms (227 ± 57 and 216 ± 97 vs. 135 ± 117 points) (Mean ± SD), can be explained by the fact that the Mexican and Greek farms were selected on the basis that they had to keep records of all diseases and production data. Only the "better" farmers do so, whereas the Dutch farms were selected completely random.

Cows are highly motivated to maintain lying times of 12 to 13 h/day (Jensen et al., 2005). Lying time can, therefore, be a good indicator for animal welfare or cow comfort (Fregonesi and Leaver, 2001), but it takes a major time investment to measure it. It is therefore, that in this system is chosen to evaluate the conditions that are required for lying and known to promote lying in cattle. This is much more practical. Overcrowding is one of the known factors that will reduce lying time (Fregonesi et al., 2007). A comfortable bedding will increase lying time (Rushen et al., 2001), but also the size of the free stalls and type of divider are of importance (Tucker et al., 2004; 2005). An indication for the lying time can be derived from the number of cows standing idle. This is, however, depending on the time of the day and other factors as well. During lying the blood flow though the udder is 25% to 50% higher and this will result in a higher milk yield (Metcalf et al., 1992).

The scoring system was used by many persons and on many farms. After a short training, all observers could evaluate a farm in less than 1 hour, if the farmer had the historical health data ready. So it is a system that can be implemented in the routine of herd health consultants. Because it is numerical, one can compare the comfort level between farms worldwide. The Welfare Quality® system takes about 1 day for 1 farm and does not result in a numerical score, which makes it more complicated to use in comparative studies. Despite the duration difference in execution, the results of both systems had a correlation of 0.84 ($P < 0.01$). One could save more time by just counting the number of cows standing idle, which is also known as the cow comfort index as proposed by Cook et al. (2005). The correlations with the total score were not that high, however. But with the Welfare Quality system it was 0.87 ($P < 0.01$). The extra information obtained by executing the entire protocol of that system is thus costing a great deal of time.

It is important to realize that negative scores weigh more than positive ones, conveying strength to this system. Other systems that evaluate animal welfare status, such as the Animal Needs Index (Ofner et al., 2003) and Welfare Quality®, weigh certain parameters more than others, but never depending on the score of

that parameter. However, if a certain aspect of welfare, *e. g.* food, is negatively scored, this implies that there is a need for that particular aspect. If an animal is hungry, food is the main thing that occupies his/her mind at that moment. The search for food is dominating other needs, like proper bedding or social contact. With a full belly, proper bedding and social contact become, relatively, more important. If a cow has mastitis, she will feel bad. Having access to pasture is less important then since the animal just wants to get rid of the disease. It is therefore that in the presented system a minimum score needs to be acquired for each chapter. If the minimum score is not reached, the difference between the score for that chapter and the minimum is subtracted from the total. This characteristic will allow for this parameter to stand out in comparison with the total score. The use of this scoring system in three different countries, in a wide variety of farms, demonstrate that the scoring system is reproducible and user friendly in order to assess the welfare status of the cows on a dairy farm. The practical execution of the cow comfort scoring system is substantially less time consuming and easier to perform than the Welfare Quality® assessment protocol.

(An Excel sheet with formulas can be obtained via email F. J. C. M. vanEerdenburg@ uu. nl)

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Animal Welfare and Ethical Implications in Finfish Aquaculture

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Summary: The ethical implications of aquaculture, regarding fish welfare and environmental aspects were investigated. The aquaculture industry has grown substantially the last decades, both as a result of the over-fishing of wild fish populations, and because of the increasing consumer demand for fish meat. As the industry is growing, a significant amount of research on the subject is being conducted, monitoring the effects of aquaculture on the environment and on animal welfare. Although the existence of fish sentience is still debated, most biologists are at present acknowledging the probability of fish being sentient creatures of welfare concern. The areas of concern when it comes to animal welfare have here been divided into four different stages: breeding period; growth period; capturing and handling; and slaughter. Current aquaculture practices are affecting fish welfare during all four of these stages, both on physical and mental levels, as well as on the ability of fish to carry out natural behaviours. The effect fish farming has on the environment is here separated into five different categories: the decline of wild fish populations; waste and chemical discharge; loss of habitat; spreading of diseases; and invasion of exotic organisms. There is evidence of severe negative effects on the environment when looking at these five categories, even when considering the difficulty of studying environmental effects, due to the closely interacting variables.

Introduction

The amount of fish killed for food each year is steadily increasing, as the consumer demand for fish, as well as the world's human population, is growing rapidly. Through unsustainable fishing of wild populations of fish, the number of fish reared in farms has increased greatly the last decades. From previously answering for 9% of the total of fish killed for human consumption in the middle of the 1980ies, aquaculture systems produced 43% of the consumed fish in 2006 [9], and will soon be answering for the majority of fish killed for meat worldwide [5]. The rising consumer demand for high quality fish meat, and at the same time sustainable fish farming concerning animal welfare and the environment, has led to an increased interest in aspects regarding improvements of fish farm practices [15]. In addition, the increasing public concern regarding fish welfare in aquaculture has resulted in a substantial amount of research the last decade [12].

The aim of this theoretical investigation was to review and discuss the potential risks of aquaculture practices, particularly in regards of fish welfare, but also concerning the environmental impacts of these practices.

Aspects of animal welfare

The existence of pain and suffering in fish has been widely studied and debated in recent years, with some conflicting results. If considerations should be made in aspect of fish welfare, they need to be regarded as sentient creatures, i. e. creatures with physiological and psychological characteristics that enable them to feel for example pain, fear and psychological stress. Their reactions to stimuli would, therefore, not only be a physiological response but also cause an associated psychological experience that requires cognitive capabilities [3]. It seems clear that the relatively primitive brain structures of the limbic system are to some extent involved in emotional and cognitive responses [3], and the fact that fish neuro-anatomy includes the limbic system is an indicator that it is at least a possibility that fish are capable to feel aversion to noxious stimuli. Huntingford and co-workers [12] states that it should be possible for fish to experience the subjective mental state of pain or suffering without the neocortex brain structure. Generally, the contributors to this discussion have drawn the conclusion that since the similarities between fish and mammals do not only include some aspects of neuro-anatomy, but also other anatomical and physiological structures and responses, as well as behavioural similarities, fish are creatures that should be taken into consideration regarding animal welfare and humane treatment [12, 15, 16, 21].

The potential risks for impaired welfare in farmed fish caused by humans occur under different phases of the production.

a) *Breeding*. The natural spawning can be intervened in aquaculture by procedures that enhance the gonad maturation process. This can either be done by altering the photoperiod, or by hormonal injections [8], which can have impact on fish welfare. Breeding fish for productive features, such as fast growth and high feed

conversion efficiency, has had some unexpected welfare consequences. Due to genetic and/or environmental factors, conditions such as deformities of heart, swim bladder, spine, jaw, and lips are not uncommon in farmed fish, and impair welfare through poor health and impaired swimming ability, as well as capacity to compete for food [1, 14]. Affected fish show decreased stress resilience and have increased mortality rates compared to others [1], indicating impaired welfare.

b) *Growth period*. The intense rearing conditions commonly seen in commercial fish farms is often causing poor water quality, leading to increased aggression and disease susceptibility; reduced growth, stress and distress; and mortality [13]. This is certainly a concern for fish welfare and closely interacts with other issues of concern.

c) *Capturing and transportation*. Fish are mainly captured and transported when transferred to pens for growth or in connection to slaughter. Although the duration of these stages are comparably short to the growth period, they are vital stages in regards of welfare, since they can cause numerous potential stressors for the animals. Examples of such are crowding, handling during capture, poor water quality with low oxygen levels, food deprivation, and increased aggression and spreading of diseases. Continuous or multiple stressful situations for fish are additive and exhaust the animals, making them less capable to handle further stressors [1, 10].

d) *Slaughter*. The moments leading up to death for fish in aquaculture may be short in terms of life span, but is often associated with severe stress and pain for the animals. Following methods of slaughter is currently being used on farmed fish: asphyxia, carbon dioxide narcosis, ice stunning, spiking (though used mostly on an experimental level), knocking, exsanguination through gill cutting or decapitation, salt bath in combination with evisceration (i. e. gutting), and electrical stunning [15, 20, 21]. With certainty, handling, stunning and slaughter are highly stressing moments for farmed fish whichever method being used, and the more stressful it is the lower meat quality, which makes it measurable [20]. Poli and co-workers [20] viewed the relative fast working methods of some types of stunning (e. g. knocking and spiking) generally as less stressing for the fish than other methods. The stress levels of fish before slaughter can be so severe, due to pre-slaughter procedures, which the positive effect of a more humane method of stunning no longer shows any significant differences in physiological measuring [20]. Therefore, although slaughter methods are important for fish welfare purposes, cautious handling in the capturing and pre-slaughter procedure cannot be over-emphasized.

Aspects of environmental impact

The environmental impact that aquaculture means includes:

a) *Decline of wild fish populations*. Large amount of wild caught fish is used as feed, which threatens the wild populations [6, 14, 17]. The drastic decline of wild fish in the world's oceans is not just a moral issue in itself, but also a critical environmental issue, since fish play a vital protective role of their ecosystems [11]. To explain this, Naylor and co-workers [17], point out that wild fish are being used as feed, in form of fish meal and fish oil, in aquaculture as well as other forms of meat production, and fish from intensive systems usually have been fed fish protein that equals 2-5 times the fish protein produced on-farm. Carnivorous fish species are commonly farmed and are in the western society more demanded than herbivorous or omnivorous species, making the decline of wild fish populations accelerate [18].

b) *Waste and chemical discharge*. There are various chemicals used in aquaculture practices that can affect the environment if discharged to surrounding water bodies, including different types of pesticides, fertilizers, disinfectants, antibiotics, and oxidants. These can be harmful to natural waters through eutrophication from nutrients or pollution from heavy metals, as well as influencing through medicinal or toxic impact on wild aquatic animals [2, 18].

c) *Habitat destruction*. The usage of natural habitats for wild fish as sites for aquaculture can have detrimental effects for wild fish, as well as the environment. It is commonly vulnerable coastal habitats, e. g. mangrove forests, which are being exploited [17].

d) *Spreading of diseases*. Diseases spreading between wild and farmed fish are problematic from an environmental point of view in the sense of threats to fish populations. The spread of pathogens often originates from wild fish, though due to crowding and intensity, it can often turn into epidemics when spreading to fish farms [18].

e) *Invasion of exotic organism*. More than once, exotic species of fish have competed out the indigenous fish, resulting in exotic fish displacing other species, leading to a decrease in biodiversity [7]. The most common type of farmed salmon, the Atlantic salmon, frequently escapes and constitutes about 40% of the wild fish caught of this species in the North Atlantic Ocean [17], and outnumbers Atlantic salmon with wild origin returning to streams by far [19].

Conclusions

The current issues of ethical concern in aquaculture practices are related to animal welfare, as well as,

environmental issues. Considering the challenges aquaculture would be facing, if attempting to resolve the issues of ethical concern, resolution seems essentially unattainable. The economical and nutritional gains of fish farms are not comparable with the vast environmental and animal welfare losses. In addition, the aquaculture industry has not been able to solve the issue of decline of wild fish populations, but is instead further exacerbating the decline. The ethical arguments and scientific evidences here reviewed have not all come to the same conclusions. What can be concluded, however, that the general agreement is that current aquaculture practices are neither meeting the needs of fish nor environment. Thus, it does not seem unreasonable to state the obvious environment and animal welfare aspects make it doubtful to promote increased fish consumption.

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AquaDucT Bell Drinker for Pekin Ducks—Enrichment for the Ducks Environment

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Summary: The drinking water supply of Pekin ducks is usually provided exclusively via nipple drinkers. For ducks as animals with high affinity to water, open drinking systems must be seen as enrichment of their environment, among other aspects, through the possibility of general occupation with water.

In the studies performed since 2003, different drinking systems have been tested which allow plunging of the head and sprinkling of the plumage. During pre-tests, a modified round drinker proved as an appropriate water supply for Pekin ducks. This drinker, now called "AquaDucT bell drinker" (Big Dutchman GmbH, Germany), afterwards was applied in the practice under field scale conditions with common animal numbers (herd size: 7,100 – 13,500 animals / farm, 3 farms in total). During each fattening trail several parameters were measured twice: behaviour observation, health parameters (e.g. plumage quality, the degree of soiling, obstruction of the nostrils, foot pad hyperkeratosis and necrosis), water quality of the offered drinking troughs (drip pans, nipple drinkers, bell drinkers) for microbiological tests (total germ count, number of Enterobacteriaceae) and the levels of dust and ammonia were measured.

The results concerning animal behaviour, health and hygiene of drinking water confirmed the results of the pre-test. With regard to the germ counts it has to be noticed that the total germ counts of the drip pans that are still existent in duck farming were significantly higher than in bell drinkers and nipple drinkers. The general argument that the use of open water drinking systems is detrimental to animal welfare and health therefore cannot be confirmed. The AquaDucT drinkers are accepted well by the ducks and represent a considerable improvement of their environment. These drinkers allow the animals species-specific drinking and straining, beak cleaning, cleaning of nostrils and eyes as well as the plumage which is reflected by a high activity at the drinkers.

Introduction

Domestic Peking ducks (*Anas platyrhynchos*) kept for fattening purposes are nowadays mainly reared indoors without any access to open water for labour, hygiene, technical and economic reasons. These ducks still show a clear preference for open water and make use of water for foraging and feeding, drinking, for general exploration, locomotion and peening, even without prior experience, just like their wild ancestors. These housing conditions significantly restrict their freedom to display their natural behaviour. An obvious consequence of this restriction is a deteriorated plumage condition, especially with regard to cleanliness (KNIERIM et al., 2004). Ethologists and animal rights groups criticise that the fattening of aquatic birds impedes the well-being and species-appropriate behaviour of those animals The Standing Committee of the European Convention for the Protection of Animals kept for farming purposes (1999) states that Pekin ducks need access to bathing water. Where this is not possible, access to water needs to be provided to ensure that ducks can cover their head with water, take in water with their beaks, pour water over their heads and dip their heads under water. The aim of this study was to find an alternative solution for water supply which is both animal-friendly in regards to behaviour and health and also economically feasible.

Therefore the AquaDucT bell drinker (Big Dutchman GmbH, Vechta) was tested under field conditions.

Animals, material and methods

In the studies performed since 2003, different drinking systems have been tested which allow plunging of the head and sprinkling of the plumage. During pre-tests, a modified round drinker proved as an appropriate water supply for Pekin ducks. This drinker, now called AquaDucT bell drinker (Big Dutchman GmbH, Germany), afterwards was applied in the practice under field scale conditions with common animal numbers (herd size: 7,100 – 13,500 animals / farm, 3 farms in total). At the participating farms, five to eight alternating control trials [solely nipple drinkers (ND)] and test trials [additional offering of bell drinkers (BD)] were carried out. The bell drinking system was installed on the incline side of the stables and was available to the ducks for six

hours daily during a test trial starting the 25th day of life.

During the study each fattening trail was visited twice (28th to 32nd and again 35th to 39th day of life) and several parameters were measured: behaviour observation, health parameters (e. g. plumage quality, the degree of soiling, obstruction of the nostrils, foot pad hyperkeratosis and necrosis), water quality of the offered drinking troughs [nipple drinkers with/or without drip pans (DP), bell drinkers] for microbiological tests (total germ count, number of Enterobacteriaceae) and the levels of dust and ammonia were measured.

Results and discussion

Related to the dust and ammonium concentrations (mean values ± SEM), in none of the farms the type of visit (control or test trail) or the time of the visit (1st or 2nd) had a significant influence on dust or ammonium levels. The measured dust concentrations varied, regardless of the farm, between 0.53 ± 0.01 mg/m^3 and1.08 ± 0.21 mg/m^3, the noxious ammonia were measured between 4.33 ± 1.21 ppm and 8.76 ± 0.24 ppm (see Table 1). The dust levels in poultry houses quoted by PETERMANN (2006) with mean concentrations of 3.60 mg/m^3 and the dust levels in duck houses recorded by ZUCKER et al. (2005) with mean concentrations of 1.4 mg/m^3 were not reached during this work either in control or test runs.

The mean ammonia concentrations were below the recommended 10 ppm at all times and therefore meet the requirements of the Order on the Protection of Animals and the Keeping of Production Animals (German designation: Tierschutz-Nutztierhaltungsverordnung, TierSchNutztV).

Table 1 Survey over the mean dust (mg/m^3, MW ± SEM) and ammonia concentrations (ppm, MW ± SEM) in the three farms during control and test trails and the first (28th to 32nd day of life) and second (35th to 39th day of life) visit during the fattening periods ml (Description of the bioburden: n = number of samples, SEM = Standard Error of the Mean, SD = Standard deviation)

Farm	Type of trail	Visit	Dust			Ammonia		
			n	MW ± SEM	SD	n	MW ± SEM	SD
1	Control	1st	8	0.90 ± 0.20	0.58	7	6.71 ± 1.26	3.33
		2nd	8	1.08 ± 0.21	0.61	8	6.99 ± 1.38	3.91
	Test	1st	8	0.55 ± 0.05	0.15	7	6.13 ± 1.29	3.42
		2nd	8	0.68 ± 0.11	0.31	7	5.00 ± 0.25	0.66
2	Control	1st	5	0.54 ± 0.11	0.26	4	4.33 ± 1.21	2.41
		2nd	5	0.74 ± 0.16	0.37	5	5.89 ± 0.48	1.07
	Test	1st	5	0.53 ± 0.09	0.19	5	7.78 ± 1.83	4.09
		2nd	5	0.71 ± 0.15	0.32	3	8.76 ± 3.19	5.52
3	Control	1st	8	0.81 ± 0.15	0.42	6	7.58 ± 1.45	3.56
		2nd	8	1.08 ± 0.15	0.42	5	7.07 ± 1.59	3.55
	Test	1st	8	0.71 ± 0.11	0.32	7	7.17 ± 1.37	3.61
		2nd	8	0.96 ± 0.19	0.53	7	6.61 ± 1.33	3.51

The average total germ count and the number of Enterobacteriaceae (113 ± 30 CFU/ml, n = 187) were lowest at the nipple drinking system, followed by the AquaDucT bell drinkers (Enterobacteriaceae 14.763 ± 2.459 CFU/ml, n = 33) and the drip pans with the highest amount of total germs and enterobacteriaceae (47.301 ± 11.057 CFU/ml, n = 44). For details relating to the average total germ count see Table 2. With regard to the qualitative analysis of the samples for salmonella, it was possible to isolate salmonella from all offered drinking systems (number of sample with salmonella: ND = 1; DP = 9; BD = 5).

Although the total germ count was always higher at the AquaDucT bell drinkers than at the nipple drinkers (but lower than at the drip pans), a negative influence on the health of the Pekin ducks could not be observed. The investigated control animals did not differ significantly from the test animals regarding their IgY-content. A rise of the total loss rate in percent was not recognizable.

Table 2 Farm independent survey over the average total germ count in CFU/ml (Description of the bioburden: n = number of samples; ND = nipple drinkers, DP = drip pans (one farm), BD = bell drinkers, SEM = Standard Error of the Mean)

	ND	DP	BD
n	226	62	40
MW ± SEM	10,950 ± 1,583	5,174,412 ± 564,137	3,955,864 ± 877,640
Median	6100	3,750,000	2,251,875
Min	75	900,000	372,750
Max	260,000	27,900,000	25,525,000

The results concerning animal behaviour, health and hygiene of drinking water confirmed the results of the pre-

test. All results show that Pekin ducks clearly preferred the modified bell drinkers "AquaDucT" over the nipple drinkers. They allow the animals to dunk their heads, to drink and strain the water in a species appropriate manner, to groom their plumage with water and to clean their beaks and eyes.

During the testing phase, the drinking activity ("drinking" and "cleaning in the drinking area") increased significantly ($P < 0.001$) up to 90% during the period of access to the round drinkers, whereas the nipple drinkers were used less during this period and considerably fewer animals rested. After the bell drinkers were raised, the activity level at the nipple drinkers on this side increased. On the side of the nipple drinkers, the lowering of the bell drinkers had no influence on the behavior of the ducks. During the course of the fattening, drinking behavior increased in all trials. A modified form of bathing behavior could be observed at the bell drinkers where ducks scooped water onto their plumage with their heads, and then interrupted this routine to clean their plumage. A differentiation of the originally described cleaning with drinking water could not be made in the video evaluation.

Also, in the assessment of the animal health, the ducks with access to bell drinkers almost always scored significantly better ($P < 0.05$). The ducks in the test trials had cleaner plumage, less obstructions of the nostrils and patency of the nasal cavity and fewer eye infections than the animals that solely had access to nipple drinkers.

Conclusions

The modified bell drinkers are accepted well by the ducks and represent a considerable improvement of their environment. These drinkers allow the animals species-specific drinking and straining, beak cleaning, cleaning of nostrils and eyes as well as the plumage which is reflected by a high activity at the drinkers. As a result, the bell drinker AquaDucT provides a good water source for Pekin ducks. These drinkers could represent a possibility to fulfil the recommendations of the Standing Committee of the European Convention for the Protection of Animals kept for farming purposes concerning Peking ducks (1999).

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The Impact of Staying on the Pasture on the Occurrance of Acts and States of Comfort Behavior in Horses Kept in Stable

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Summary: The movement is a very important factor in the life of the horse and its restriction can adversely affect the health of the animal. The aim of this study was to compare the occurrence of acts and behavioral states in the horse attending the pasture regularly for 4 – 7 hours / day with the horses leaving the stable for training only. The study included eight adult warmblood horses used in sports. The horses staying in a stable remained there for at least three weeks before the investigation had begun. Observations lasted 6 days and 15 hours of each day were considered. The included behavioral states included sternal recumbency, lateral recumbency, occupation with the bedding material, eating and standing. The behavior acts included rolling, gnawing objects in range of a horse, kicking the walls and walking around the box. Horses attending the pasture exhibited statistically significant ($P < 0.01$) longer mean time for staying in one place and less time for occupying with the bedding. The average length of recumbancy for both sternal and lateral did not differ significantly, but the higher frequency of ling down behavior was observed in horses leaving the stable only for the training. The frequency of all observed behavioral acts differed significantly ($P < 0.01$) between the two investigated groups. Horses attending pasture were less often rolling, gnawing objects, kicking the walls and walking around the stall box. In this study it was found that attending the pasture for 4 – 7 hours a day has a positive effect on horses mental state and leads to a lower incidence of adverse behavior in the stable.

Introduction

Individual housing of horses in the stables may be preferred by some owners in order to reduce the risk of injuries that occur as a result of the free movement and interaction between individuals [8]. This approach to management practice, especially as it comes to valuable and therefore expensive animals, is found in particular in developed countries, and becomes more and more common [9]. Owners of animals housed this way believe that training is sufficient to meet the needs of the movement of horses, and that horses in the stalls being separated by grill walls are provided with enough of social contact.

The aim of this study was to compare the occurrence of acts and behavioral states evincing the comfort of working horses housed in single stalls and leaving the stable only for the training with horses leaving the stable daily also for free exercise.

Material and methods

The study was conducted in a stable, located in the north-eastern Poland in the period from 2nd November until 8th November, 2011. The stable contained nine stalls (2.95 × 3.40 m) in which 8 horses were placed. All animals were adult and were used for sports (show jumping). For the behavioral observations horses were divided into 2 groups with 4 animals in every group. Horses from the first group (S) remained in a stable (at least three weeks before the start of the observation) and were leaving it only for training, the horses of the second group (P) were regularly leaving the stable for free exercise on pasture (4 – 7 hours / day). Observations were carried out for 6 days on a continuous basis. The horses' behaviour in the stable was observed continuously via video camera (DC max D-C-500 F) recordings (18:00 – 9:00). The frequency and total duration of the behaviour patterns including, behaviour states: sternal and lateral recumbency, standing at rest, occupation with bedding material, and the frequency of behavioural acts: rolling, pica, kicking stall walls and locomotion (walking in circles) inside the stall as well the frequency of reclining to sternal and lateral recumbency were observed. The values of the investigated traits were processed statistically using Statistica 10.0 PL software. To determine the significance of differences between means analysis of variance in univariate orthogonal system and a multi-Duncan test was used.

Results and discussion

All behavioral acts and states were observed in horses leaving the stable only for the training (group S). None of the animals from group P showed the pica behaviour in the stable. The average length of the standing at rest performed by horses from group S was 812 minutes, and by the horses from group P was 1130 min ($P < 0.05$) (Table 1).

Horses remaining in the stable (S) spent longer time

on occupation with bedding material in their stalls (approximately 60 minutes) than horses from group P, which were leaving stable for free exercise (46 min) ($P < 0.05$). There were no statistically significant differences in the length of staying in sternal and lateral position between the two groups, but the mean time of horses from group P was noticeably shorter than of the horses remaining in the stable (S). Horses from group P ate longer after giving them the feed (mean 176 min) compared with the horses from group S (170 min), but this result was not statistically significant (Table 1). The statistically significant differences ($P < 0.05$) were found for the following behaviors: rolling, frequency of lying down in sternal position, standing at rest and a highly significant differences ($P < 0.01$) were found for: lying down in lateral position, pica, kicking the stall wall, walking in circles. Horses from group P showed rolling behaviour more often (28 times) than the horses from group S (4 times), but they were less frequently kicking the stall walls (2 times-group P, 74 times-group S) and walking in circles (572 times the horses from group P and 704 horses of group S). In addition, the horse from the group P did not show pica behaviour whereas horses from the group S manifested this type of behavior 48 times.

Table 1 Mean duration and frequency of behavioural states and acts

Behavioural states (min)					
Group	Sternal recumbency	Lateral recumbency	Occupation	Feed intake	Standing
S	38	24	60^{a}	170	812^{a}
P	36	22	46^{b}	176	1130^{b}
Comfort acts					
Group	Sternal recumbency	Lateral recumbency	Rolling	Standing	
S	116^{a}	78^{A}	4^{a}	290^{a}	
P	116^{b}	26^{B}	28^{b}	304^{b}	
Undesirable acts					
Group	Pica	Kicking	Locomotion		
S	48^{A}	74^{A}	704^{A}		
P	0^{B}	2^{B}	572^{B}		

A, B = highly significantly different ($P < 0.01$); a, b = significantly different ($P < 0.05$).

Based on fossil evidence, postures and movement patterns of equids have changed as a result of evolutionary changes in body and limb morphology [10]. Besides lengthening of the limb bones and changes to prevent lateral movement of joints, the development of the so-called spring ligaments was significant, and very important for locomotor activity on a hard ground during wandering long distances. The locomotor activity still remains a fundamental behavior for each horse, and therefore modern horses require a large amount of it [3, 7, 10]. It is believed that the horses without possibility for free exercise, show increased activity, which is the manifestation of the accumulation of unspent energy. Our study confirms this view. In addition, in case of riding horses, while standing, their front legs carry about 60% of the total weight of the animal. During locomotor activity the ratio changes even more, and the load is even bigger when adding also the effect of centrifugal force appearing on curves. All these loads are taken by the forelegs. Therefore, the increased frequency of locomotor activity of horses around a small box (area of 10 m^2 to 500 kg horse corresponds to a dimension of 1.2 m^2 for a man of 70 kg body weight), with tight curves can lead to congestions and wear and tear of the limbs, leading to their injuries [1]. Our study have shown a statistical highly significant differences in the frequency of walking around the stall by horses. Horses from group S walked around more often than the horses from group P (Table 1).

Our study also included observations concerning the resting behavior. Horses rest periodically, where they cease all activity and become quiescent [10]. Adult horses often rest in a standing posture. However, because while resting in this posture horse's sense of balance has to work, it must be assumed that the resting in standing position is not as intense as in a recumbency [2]. That is why horses recline to recumbency at least once a day, if environmental conditions are favorable for doing so. Our study have shown that the length of the horses' standing still in group S was shorter (Table 1) and the fact could indicate a higher degree of their nervousness and anxiety. These results are consistent with the results of Warhahn'a et al (2011) [9].

Sleep is of great importance in the life of every creature. Horses exhibit different states of sleep: drowsiness, slow-wave sleep and paradoxical sleep. Slow-wave sleep is initial and more frequent form of equine sleep and can occur in standing or recumbent position; it occurs before each bout of paradoxical sleep. Paradoxical sleep is a very deep sleep occurring in lateral or occasionally sternal recumbency. Oswald (1969) [4] suggests that the main function of slow wave sleep is for

body restitution, while paradoxical sleep may be mainly for brain repair. The study conducted by Pedersen et al. (2004) [5] showed that paradoxical sleep deprivation in horses leads to their anxiety, irritability, and difficulty with concentrating. Therefore, it is very important to provide proper conditions for the animals to rest and sleep. The obtained results in our study do not indicate a statistically significant differences in the length of recumbency between the groups, in both postures: sternal (36 min gr. S min and 38 gr. P) and lateral (24 min gr. S min and 22 gr. P). However, the frequency of reclining to recumbancy in both groups differed statistically significantly (Table 1). Horses from group S, compared to the horses from group P, more often reclined to sternal recumbency ($P < 0.05$), as well as to lateral recumbency ($P < 0.01$). This situation may arise from the fact that in the new, or not certain environment, horses often do not lay down. This is a situation that the horse seems to be uncertain of and psychological factors matter. In this case, the standing posture is atavistic behavior, inherited from ancestors who, for example due to threats by predators had to stay alert, sometimes staying for a long time in the standing position [2]. Perhaps the horses staying in the stable feel more confident in its environment. Longer time spent on occupation with bedding (difference between groups statistically significant $P < 0.05$), more frequent pica and more frequent locomotor activity (differences statistically highly significant $P < 0.01$) manifested by horses from group S, may indicate their willingness to release the unspent energy resulting from boredom due to lack of exercise and stimulus-poor environment [9]. Longer time of the feed intake by horses from group P may indicate their higher calmness and lower nervousness. In group S higher incidence of undesirable behavior such as kicking in a stall walls was observed. This confirms the results presented by Rivera et al. (2002) [6], who found that foals kept in stables develop more undesirable behaviors, including kicking walls of the stalls, than horses kept in groups on the paddocks. Higher frequency of rolling, exhibited by the horses of the group P may be associated with their higher frequency of lying down and getting up. According to Waring (2002) [10] repeated rolling occurs after a period of recumbency. This action may be performed several times before standing up.

Conclusions

The study has shown that the housing system of horses and enabling them to fully manifest their behavioral needs is an important factor influencing the behavior of the horses in the stable. The owners claiming that not letting their horses out to pasture will protect them from injury is not correct and should not be tolerated. It seems that in fact preventing horses from social contacts and impossibility of discharging unspent energy can lead to frustration and the occurrence of behaviors leading to injuries (such as kicking the stall walls). Furthermore, the study indicates that training does not fulfill the horses' exercise requirements.

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A Model of Evaluation of Milk Cows Welfare by Nutritional-Metabolic Indicators

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Summary: The way in which the welfare is considered, varies very much from a concept to another, from a specialist to another; until now there isn't a standard method for its measurement. The welfare is equally influenced by conditions of nutrition and watering, environment and informational circuit between animals and their environment. For the same conditions of life, the animals have the different answers and methods of accommodation, there being the differences between species, breeds, ages, genders, physiological statements and individuals.

The scope of this study is to develop a model for evaluation of animal welfare, in the first instance for farm animals, by scientific indicators, based by results obtained in lab, by analysis of biological products (blood, urine, milk) taking from animals, using the method of *metabolic profile* in addition with analysis of water, feed and microclimate parameters. By this analysis, we want to complete the requirements prescribed in EU legislation, based on rigorous statistical analysis of data obtained by hematological and biochemical tests in comparison with the reference values for a number of 20 parameters performed in 100 milk cows, before and after calving.

We wants that this model, following a *multi*-criteria approach, to serve as base for communication between the different interested parts: farmers-veterinarians-consumers, in order to assure the animal welfare and health and security of food and environment.
Key words: animal welfare, milk cows, metabolic profile, environment security, food safety

Introduction

The *method of metabolic profile* has special position among the laboratory's methods for diagnosis of nutritional-metabolic diseases. In this project, we wish to promote it as method of evaluation of animal welfare in "*alive*" animal.

The "*metabolic profile*" means the evaluation of nutritional-metabolic integrity of livestock, by performing of biochemical and haematological exams. It permits rapid, preclinical recognition of nutritional-metabolic disorders, giving it the capacity of continuous metabolic surveillance of big livestock, with rapid taking of measures for adjustment of nutrition and assurance of welfare in the same time.

Material and methods

The analyses were performed in the National Reference Laboratory for Assessment Animal Welfare from Institute of Diagnostic and Animal Health Bucharest, Romania.

All the analytical methods are accredited by the national organization for accreditation RENAR.

During May-September 2011, there were examined 100 blood samples from cows of Frise Holstein breed, different ages and physiological states.

The samples were sent for analysis-*making of the metabolic profile* -by the from milk cow farms situated in plain areas of the country. The animals were kept in a summer camp, without having the adequate (necessary) space for sheltering and the external temperatures reached 40℃ degrees; their fodder was made up of combined granulated forage for milk cows being composed of: soy protein (GMO), sunflower protein, by pass soy, cereals, phosphate mono-calcium, calcium carbonate, premix vitamin-mineral and salt.

The blood was taken by puncturing the jugular vein, in *Vacutainer* tubes, in the morning, before the feed administration.

For making the hematological examination, the blood was taken in vacutainers with anticoagulant (EDTA) ×3 ml-e. g. *quantitative hematological* at the automatic analyser AcT5diff CP, e. g. *qualitative hematological* (leucocytes formulae and cyto-morphological examination on smear of peripheral blood coloring May Gr nwald Giemsa, at the microscope Leica DM LS2).

For the biochemical examination, the blood samples were taken in vacutainers without anticoagulant, the blood was left at room temperature in order to favor the clot retraction and the serum highlight, using either classical biochemical methods (spectrophotometry) and modern methods-analyser VETSCAN VS2.

In this study, we evaluated the following profiles:

a) *hematological profile*: RBC, hemoglobin, hematocrit, eritrocyte indices derived (MCV, MCHC), WBC, leucocytes formulae and cyto-morphological examination;

b) *proteic profile*: total protein, albumins, globu-

lins, urea;

c) *energetic profile*: glycemia;

d) *mineral profile*: calcium, phosphorus, magnesium;

e) *vitamin profile*: vitamin E, β-carotene;

f) *enzyme profile*: aspartat-amino-transferase (ASAT), gama-glutamil-transferase (GGT)

Result and discussion

By comparison with the reference normal physiological values for breed, different ages and physiological states, we obtained the following results which are represented in the Table 1.

The rest determined hematological and biochemical parameters were had the normal values for species, breed, age and physiological states of investigated animals.

By correlations of obtained results, we observed:

– *mycrocitic anemia normochromic*, easy *leucocytosis* with eosinophilic and neutrophilic reaction, *hypo*-glycemia, *hypo*-proteinemia and marked *hypo*-beta-carotenemia, hepatopathy with severe phenomena of extra-hepatic obstruction (cholestasis), in pregnant cows;

– marked *mycrocitic anemia normochromic*, *leucocytosis* with lymphocytic, neutrophilicand eosinophilic reaction, *hypo*-proteinemia, *hypo*-phosphoremia, *hyper*-magnesiemia, marked *hypo*-beta-carotenemia and hepatopathy with severe phenomena of extra-hepatic obstruction (cholestasis), in dairy cows at 0 – 21 days after calving;

– *mycrocitic anemia normochromic*, easy *leucocytosis* with neutrophilic and lymphocytic reaction, *hypo*-glycemia, *hypo*-proteinemia, *hypo*-calcemia *hypo*-phosphoremia, *hyper*-magnesiemia, marked *hypo*-beta-carotenemia and hepatopathy with severe phenomena of extra-hepatic obstruction (cholestasis), in dairy cows at 22 – 45 days after calving;

– these paraclinical *pictures* can be the consequence of some states of nutritional-metabolic stress induced by thermal discomfort, translated by the decrease of productivity, disorders of reproduction, raised mortality in newborns, weaning youths and even mortality in adult animals;

– the disorders in protein malnutrition in natural conditions must not be regarded as pure mono-deficiencies but, as *multi*-deficiencies, determining a perturbation in vitamin assimilation and a reduction of all the metabolic processes. The oxide-reducer processes decrease as intensity; anemia, tissue atrophy, anorexia, reduction in diseases resistance and loss of weight are installed.

Table 1 Nutritional-metabolic indicators model of evaluation of milk cows' welfare

	Hematological examination		Biochemical examination				
	***Quantitative* hematological examination**	***Qualitative* hematological examination**	**Energetic profile**	**Protein profile**	**Mineral profile**	**Vitamin profile**	**Enzyme profile**
Pregnant cows (9^{th} month) (14 – 21 days before calving)	↓ HCT: 33.7% – 28.1% ↓ MCV: 53 – 44 fL ↑ WBC: 10.4 – 12.9 × 10^3/μL	↑ Neutrophils: 52% – 70% ↑ Eosinophils 8% – 14% Anisocytosis + + Poikilocytosis + + Atyp. Lymph and "*nude*" cells + +	↓ Glycemia: 36 – 20 mg/dl	↓ Total protein: 7.0 – 6.42 g/dl		↓ β Carotene: 112 – 77 μg/dl	↑ GGT: 28 – 77 U/L
Dairy cows (0 – 21 days after calving)	↓ HCT: 31.1% – 22.4% ↓ MCV: 50 – 42 fL ↑ WBC: 14.1 × 10^3/μL	↑ Neutrophils: 52% – 68% ↑ Lymphocytes 80% – 84% ↑ Eosinophiles 12% – 16% Anizocytosis + + Poikilocytosis + + Atyp. Lymph and "*nude*" cells + +		↓ Total protein: 6.80 – 6.42 g/dl	↓ P: 5.40 – 4.40 mg/dl ↑ Mg: 3.1 – 3.0 mg/dl	↓ β Carotene: 118 – 75 μg/dl	↑ GGT: 30 – 78 U/L
Dairy cows (22 – 45 days after calving)	↓ HGB: 8.8 – 7.3 g/dl ↓ HCT: 28.3% – 19.2% ↓ MCV: 46 – 40 fL ↑ WBC: 10.8 – 12.0 × 10^3/μL	↑ Neutrophils: 60% – 72% ↑ Lymphocytes 76% – 78% Anizocytosis + + Poikilocytosis + + Atyp. Lymph and "*nude*" cells + +	↓ Glycemia: 34 – 32 mg/dl	↓ Total protein: 6.71 – 6.36 g/dl	↓ Ca: 8.60 – 8.10 mg/dl ↓ P: 5.31 – 4.75 mg/dl ↑ Mg: 3.3 – 3.1 mg/dl	↓ β Carotene: 165 – 96 μg/dl	↑ GGT: 25 – 81 U/L

Conclusions

1. These observations highlights, in all cases, the existence of nutritional-metabolic imbalances of nature protein, energetic, vitamin, mineral and the evolution of hepatic dystrophies with phenomena of cholestasis, chronic inflammations, infections produced by pathogenic germs (viruses, bacteria, parasites, fungi) and toxicosis states (*hyper*-magnesiemia) with the change of general status of animals;

2. It must be noted that the *metabolic profile* tests are indispensable for a complete diagnosis and these give it a big importance as capacity of preclinical detection and "query" of animals related by their welfare, that allows the practicing of preventive medicine on basis of tests of surveillance/monitoring;

3. It can observe once again the importance of determination of nutritional-metabolic indicators for animal welfare evaluation for guarantee of farm *bio*-economy;

4. The blood metabolic profile must be completed, in function of case, with analysis of another tissue constituents from urine, milk, colostrum, water, feed, fodder and microclimate parameters.

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Welfare Assessment using Blood Profile in Crossbred(Landrace × *Desi*) Barrows Reared on Different Floor Space Allowances

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Summary: An attempt was made to study the stress and general health based on blood profile in 36 crossbred (Landrace × *Desi*) weaned barrows reared on 3 different floor space allowances (n = 12 each). T_1 (control) group provided floor space as per Indian Standards (IS: 3916-1966) specification, while T_2 and T_3 Treatment groups with 33% and 50% reduced floor space allocation per pig in comparison to IS. During weaner (6 – 14 weeks), grower (15 – 22 weeks) and finisher (23 – 29 weeks) stages, 3 different floor spaces {T_1 group (0.9, 1.35 and 1.8 m^2/pig), T_2 group (0.6, 0.9 and 1.2 m^2/pig) and T_3 group (0.45, 0.68 and 0.9 m^2/pig)} were provided. Blood samples were collected from 5 barrows of each group during grower (18th weeks of age), finisher (24th week) and final stage (29th week) for various parameters. The average initial body weight of barrows were 8.67 ± 0.26, 9.01 ± 0.33, 9.13 ± 0.36 kg while final body weight were 81.20 ± 2.57, 82.38 ± 3.46, 81.13 ± 2.77 kg for T_1, T_2 and T_3 groups, respectively. Although final body weight of pigs did not differ significantly among groups but plasma cortisol concentration was significantly ($P < 0.05$) higher in T_3 group (119.97 ± 11.65 nM/L) when compared to T_1 group (80.75 ± 7.99 nM/L) which is suggestive of chronic stress of pigs reared in lowest floor space. However, all other physiological variables (total protein, albumin, ALP, SGOT and SGPT) did not vary indicating the normal hepatic functions and metabolism in reduced floor space groups; all values were within an expected normal range. As lowest of the allocated floor space (T_3 group) in this experiment was higher than recommended optimum floor space for pigs in Western and neighboring countries, performance of animals along with their physiological status did not alter (except plasma cortisol in T_3 group). It indicates scope of 33% reduction of floor space for pig rearing in India.

Introduction

Scientific evidence indicates that space is not as important for pigs as other resources, e. g. food availability if their minimum space requirement is fulfilled [8]. Efficient use of indoor floor space results in low capital investment in buildings and infrastructure, reduced cost of labour and bedding systems, and represent the principal economic and management benefits [1]. A recent study [2] on space use, synchronisation and clustering of behavioural activities of pigs indicated that the theoretically derived requirements on space allowance might be reduced without compromising the Comfort Class level (a specific minimal level of husbandry conditions of animals, at which welfare of animals is not compromised). European Union recommends 0.2 to 0.55 m^2 floor space per pig, ranging between 10 to 85 kg BW [10]. Whereas, IS suggests floor area (covered) of 0.9 and 1.8 m^2/pig for weaner and finisher pigs, respectively. In some countries minimum floor space recommended per pig is considerably above the EU level [5] which is also true about India. Therefore, present investigation was carried out to explore possibility of reduction in floor space allowance for Indian crossbred pigs without adversely affecting their health, so also welfare. Blood profile is used as one of the important indicator for welfare assessment of animals. The impact of floor space allocation on physiological status of pigs has been assessed in very few studies. Physiological variables in the form of blood parameters were studied to assess welfare status of pigs although reduced floor space allocations were relatively more in comparison to most of the other countries.

Material and methods

The experiment was conducted at the Swine Production Farm, Indian Veterinary Research Institute, Izatnagar, India, between May and November months of year 2012 i. e. during summer, autumn and early winter seasons. A total of 36 crossbred {Landrace × *Desi* (local Indian)} male piglets, from 14 litters of unrelated sows farrowed contemporarily, were selected randomly taking body weight and age into consideration. These piglets were castrated at one month of age, weaned at 6 weeks of age and subsequently distributed randomly to three equal groups (n = 12 each) based upon 3 different

floor space allowances. T_1 (control) group provided floor space as per Indian Standards [7] specification, while T_2 and T_3 treatment groups with 33% and 50% reduced floor space allocation per pig in comparison to IS. IS suggests covered floor area of 0.9 and 1.8 m^2/pig for weaner and finisher pigs, respectively. During weaner (6 – 14 weeks), grower (15 – 22 weeks) and finisher (23 – 29 weeks) stages, 3 different floor spaces {T_1 group (0.9, 1.35 and 1.8 m^2/pig), T_2 group (0.6, 0.9 and 1.2 m^2/pig) and T_3 group (0.45, 0.68 and 0.9 m^2/pig)} were provided. Under each treatment group, three units of 4 piglets each were made and these piglets were kept in independent pens with specified floor space (Table 1).

Table 1 Floor space allowance (m^2/pig) for different treatment groups

Stages	T_1 group	T_2 group	T_3 group
Weaner (6 – 14 weeks)	0.9	0.6	0.45
Grower (15 – 22 weeks)	1.35	0.9	0.68
Finisher (23 – 29 weeks)	1.8	1.2	0.9

Width of each pen measured 2.5 m and specified space allocation was ensured by altering length of the pen using metallic grill gates. Floor was made of concrete with serrations to avoid slippage. Animals were fed twice daily with provision of potable water round the clock. Animals with respect to their stage were fed with weaner and grower-finisher feed as per farm's standard. Standard management practices related to health and hygiene were followed as per farm's guidelines.

Blood samples were collected from 5 barrows of each group during grower (18^{th} weeks of age), finisher (24^{th} week) and final stage (29^{th} week) for various parameters. Blood samples (5 ml) were collected in heparinized tubes. Plasma was collected and stored at −20℃. Plasma cortisol concentration was determined by standard technique using commercial radio-immunoassay kit. Total Protein and albumin were estimated using biuret method and Bromocresol green (BCG) method [3], respectively. Plasma globulin was obtained by subtraction of albumin from total protein (TP). Plasma concentration of enzyme ALP was estimated using Tris Carbonate buffer while plasma SGOT and SGPT concentrations were estimated using IFCC method.

The data obtained were subjected to one way analysis of variance (ANOVA) using the SAS software (version 12.0) package.

Results and discussion

The average initial body weight (6 week) of barrows were 8.67 ± 0.26, 9.01 ± 0.33, 9.13 ± 0.36 kg while final body weight (29 week) were 81.20 ± 2.57, 82.38 ± 3.46, 81.13 ± 2.77 kg for T_1, T_2 and T_3 groups, respectively. Although final body weight of pigs did not differ significantly among groups but plasma cortisol concentration was significantly ($P < 0.05$) higher in T_3 group (119.97 ± 11.65 nM/L) when compared to T_1 group (80.75 ± 7.99 nM/L) which is suggestive of chronic stress among the pigs reared on lowest floor space allowance. However, all other physiological variables (total protein, albumin, ALP, SGOT and SGPT) were not statistically different indicating the normal hepatic functions and metabolism among pigs of all the floor space groups. Thus, all values which are with in expected normal range indicate good welfare. As lowest of the allocated floor space (T_3 group) in this experiment was higher than recommended optimum floor space for pigs in Western and neighboring countries, performance of animals along with their physiological status did not alter (except plasma cortisol in T_3 group). A circadian pattern of total cortisol is present in many species including man, horse, and pig. For the pig this rhythm is characterized by peak amounts of circulating cortisol expressed in the morning with reduced levels during the afternoon and early evening [12]. However, values of cortisol obtained were lower than earlier study [9].

Total protein, albumin and globulin levels were within normal range as also reported in earlier study [4]. Plasma concentration of SGOT and SGPT also did not differ among the groups and values obtained were found higher when compare to earlier study [6]. Conversely, ALP concentration also did not differ among the groups and values obtained were lower than earlier study [11]. Differences in concentration of such physiological parameters when compare to other studies may be due to variation in breed of pigs, location and geographical conditions.

Conclusions

It can be concluded that there is scope of 33% reduction in floor space allowance for pig rearing in India where none of the physiological parameter was affected negatively.

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Effect of Different Floor Space Allowances on Carcass Characteristics of Crossbred (Landrace × *Desi*) Barrows

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Summary: An attempt was made to study the carcass traits of crossbred (Landrace × *Desi*) barrows (n = 36) reared on 3 different floor space allowances (n = 12 each). T_1 (control) group provided floor space as per Indian Standards (IS: 3916-1966) specification, while T_2 and T_3 Treatment groups with 33% and 50% reduced floor space allocation per pig in comparison to IS. During weaner (6 – 14 weeks), grower (15 – 22 weeks) and finisher (23 – 29 weeks) stages, 3 different floor spaces {T_1 group (0.9, 1.35 and 1.8 m^2/pig), T_2 group (0.6, 0.9 and 1.2 m^2/pig) and T_3 group (0.45, 0.68 and 0.9 m^2/pig)} were provided. The average initial body weight of barrows were 8.67 ± 0.26, 9.01 ± 0.33, 9.13 ± 0.36 kg while final body weight were 81.20 ± 2.57, 82.38 ± 3.46, 81.13 ± 2.77 kg for T_1, T_2 and T_3 groups, respectively. Six animals from each group were slaughtered as per the standard procedure. None of the major economic carcass traits {carcass weight, dressing %, back fat thickness (BFT), loin eye area (LEA),% lean etc} was affected with reduction of floor space allowances. The dressing % ranged between 73.79 to 74.6. The mean % lean value for T_1, T_2 and T_3 groups were 53.83 ± 0.69, 52.57 ± 2.36 and 53.93 ± 1.23, respectively. There was no difference in major cut-up parts as well as share of edible and inedible offal among treatment groups. Proximate composition of pork (moisture, CP and EE) also did not differ among groups. As lowest of the allocated floor space (T_3 group) in present experiment was higher than recommended optimum floor space for pigs in Western and neighboring countries, there is scope of floor space reduction for pig rearing in India. Hence, results conclude that floor space restriction did not have any adverse effect on carcass traits.

Introduction

A critical factor in the successful rearing of pigs from three weeks onwards is to have correct balance between the numbers in the group and the space allowance [3]. Scientific evidence indicates that space is not as important for pigs as other resources, e. g. food availability if their minimum space requirement is fulfilled [14]. Efficient use of indoor floor space results in low capital investment in buildings and infrastructure, reduced cost of labour and bedding systems, and represent the principal economic and management benefits [1]. A recent study [9] on space use, synchronisation and clustering of behavioural activities of pigs indicated that the theoretically derived requirements on space allowance might be reduced without compromising the Comfort Class level (a specific minimal level of husbandry conditions of animals, at which welfare of animals is not compromised). Recent [7] review on global pig production stated that weaners, growers and finishers are mostly provided about 0.36, 0.5 and 0.75 m^2/pig of floor space, respectively. Whereas, IS suggests floor area (covered) of 0.9 and 1.8 m^2/pig for weaner and finisher pigs, respectively. In some countries minimum floor space recommended per pig is considerably above the EU level [10], which is also true about India. Therefore, present investigation was carried out to explore possibility of reduction in floor space requirement for Indian crossbred pigs without any adverse effect on major economic carcass traits. The impact of space allocation on carcass back fat and percentage lean has only been reported in a few trials [5]. From the limited data available, it is not possible to predict the impact of space allocation on carcass traits, other than to state that the effect is a slight improvement in carcass lean and a slight decrease in carcass back fat depth as space is restricted with a resulting decrease in daily feed intake [4]. In present study, major carcass characteristics were evaluated with different floor space allocations, although used floor space allocations were relatively more when compare to most of the studies conducted in developed countries.

Material and methods

The experiment was conducted at the Swine Production Farm, Indian Veterinary Research Institute (IVRI), Izatnagar, India, between May and November months of year 2012 i. e. during summer, autumn and early winter seasons. A total of 36 crossbred {Landrace × *Desi* (local Indian)} male piglets, from 14 litters of

unrelated sows farrowed contemporarily, were selected randomly taking body weight and age into consideration. These piglets were castrated at one month of age, weaned at 6 weeks of age and subsequently distributed randomly to three equal groups (n = 12 each) based upon 3 different floor space allowances. T_1 (control) group provided floor space as per Indian Standards [12] specification, while T_2 and T_3 treatment groups with 33% and 50% reduced floor space allocation per pig in comparison to IS. IS suggests covered floor area of 0.9 and 1.8 m^2/pig for weaner and finisher pigs, respectively. During weaner (6 – 14 weeks), grower (15 – 22 weeks) and finisher (23 – 29 weeks) stages, 3 different floor spaces {T_1 group (0.9, 1.35 and 1.8 m^2/pig), T_2 group (0.6, 0.9 and 1.2 m^2/pig) and T_3 group (0.45, 0.68 and 0.9 m^2/pig)} were provided. Under each treatment group, three units of 4 piglets each were made and these piglets were kept in independent pens with specified floor space (Table 1).

Table 1 Floor space allowance (m^2/pig) for different treatment groups

Stages	T_1 group	T_2 group	T_3 group
Weaner (6 – 14 weeks)	0.9	0.6	0.45
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Finisher (23 – 29 weeks)	1.8	1.2	0.9

Width of each pen measured 2.5 m and specified space allocation was ensured by altering length of the pen using metallic grill gates. Floor was made of concrete with serrations to avoid slippage. Animals were fed twice daily with provision of potable water round the clock. Animals with respect to their stage were fed with weaner and grower-finisher feed as per farm's standard. Standard management practices related to health and hygiene were followed as per farm's guidelines.

Six animals (2 large, medium and small each) from each group were slaughtered at the age of 29 week (about 80 kg of average BW) following standard procedure in slaughter unit of Livestock Products Technology division of IVRI. All the animals were kept in lairage after arrival at the slaughter house and deprived of feed overnight but with free access to water. The pigs were properly stunned by using electricity and then bled by heart puncturing and subsequently wet scalding by hot water (65℃ for 5 to 6 min). The weight of hot carcass was expressed as percent of pre-slaughter live weight to arrive at dressing percentage. Lean percent of carcass was estimated using equation of [6] which includes hot carcass weight (lbs.), tenth rib fat thickness over the loin muscle (in.) and loin muscle/eye area at the tenth rib (sq. in.) as following:

Lean % = 8.588 + (0.465 × hot carcass wt.) – (21.896 × 10^{th} rib fat depth) + (3.005 × 10^{th} rib loin muscle area)

Weight of trimmed cut-up parts were measured using standard weighing balance and represented as percentage of total dressed carcass weight. Weights of different edible and inedible offal were also recorded. Samples of longissimus muscle were taken from the carcass after dissection, sealed in polythene bags and stored at –20℃ for further analysis. Collected samples were analyzed for proximate principles after thawing. Moisture, fat and protein contents of dissected longissmus muscles were determined as per the procedures of [2].

The data obtained were subjected to the to one way analysis of variance (ANOVA) as per the procedures outlined by [17] using the SAS software (version 12.0) package. The data were expressed as mean ± standard error.

Results and discussion

The average initial body weight (6 week) of barrows were 8.67 ± 0.26, 9.01 ± 0.33, 9.13 ± 0.36 kg while final body weight (29 week) were 81.20 ± 2.57, 82.38 ± 3.46, 81.13 ± 2.77 kg for T_1, T_2 and T_3 groups, respectively. Final body weight of barrows reared on different floor space allowances did not differ significantly. Average live weight (before fasting) of slaughtered barrows of T_1, T_2 and T_3 groups were 81.17 ± 5.31, 83.85 ± 6.81 and 82.43 ± 4.75 kg, respectively. Mean values of major carcass traits of economic importance were determined for slaughtered barrows of each group. None of the major economic carcass traits (carcass weight, dressing %, BFT, loin eye area,% lean etc.) was affected with reduction of floor space allowances. The dressing % ranged between 73.79 to 74.6. The mean % lean value for T_1, T_2 and T_3 groups were 53.83 ± 0.69, 52.57 ± 2.36 and 53.93 ± 1.23, respectively. Earlier finding [4] stated that very limited data was available to predict the impact of space allocation on carcass traits, other than to state that the effect is a slight improvement in carcass lean and a slight decrease in carcass back fat depth as space is restricted with a resulting decrease in daily feed intake. Previous study [15] found that restricted (0.45 – 0.74 m^2/pig) pen space for crossbred (Large White × Landrace) pigs of 10 to 23 weeks of age resulted in lower back fat measurement and dressing percentage compared to pigs that had unrestricted (0.88 m^2/pig) pen space. They suggested that reduced backfat might be due to social stress resulting from reduced pen space. [5] found that in finishing pigs (120 kg BW), back fat increased from 19.4 to 21.4 mm when available space increased from k = 0.023 to k = 0.030. Similarly, pigs were

allotted high (1.4 m^2/pig; k = 0.047) and low (1.0 m^2/pig; k = 0.033) floor space for (Landrace × Large White) × Duroc pigs of 90 – 160 kg body weight and found that BFT was higher for higher space allowance [16]. In our study, backfat and dressing % were not affected as even lowest floor space group (9 to 80 kg BW, 0.45 to 0.9 m^2/pig floor space, and k = 0.102 to 0.046 coefficient) had more space than unrestricted or high floor space of above studies. Earlier study [11] shows no differences in fat depth in pigs slaughtered at 120 kg BW and reared in restricted or unrestricted conditions (k = 0.022 versus k = 0.038), although space allowance was very low in both the groups in comparison to present study. Whereas, [8] reported that increasing space allowance was related to a decrease in fat depth, which is a highly desirable characteristic for the producer and the abattoir.

Cut-up parts (i. e. Boston butt, picnic shoulder, loin, belly, ham and jowl) were obtained from dressed carcass and their share were determined in the form of percentage of dressed carcass weight. None of the cut-up part differed significantly among the groups. Further, edible and inedible offal were also weighed and values did not differ significantly among the pigs reared on different floor space allocations. No references related to effect of floor space allowance on cut-up parts, edible and inedible offal could be cited to compare. Proximate composition of pork (moisture, CP and EE) also did not differ among groups. [13] also found that composition of the finished carcass was not affected by either stocking density (0.75 m^2 vs 0.45 m^2 per growing pig and 0.88 m^2 vs. 0.53 m^2 per finishing pig) or regrouping.

Therefore, lowest floor space allowance i. e. T_3 group (0.45, 0.68 and 0.9 m^2 for 6 – 14 weeks, 15 – 22 weeks and 23 – 29 weeks of age, respectively) for crossbred (Landrace × *Desi*) barrow had no adverse effect on carcass characteristics. It could be due to the reason that even this lowest floor space allowance is larger than optimum floor space recommended for pigs in most of the Western and neighbouring countries. Therefore, results cannot be compared directly, on the name of floor space reduction/restriction, with most of the studies where floor space allowances were very low in comparison to present study.

Conclusions

Floor space reduction (up to 50% of IS) did not have any adverse effect on carcass traits of crossbred barrows.

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Animals Welfare in Dairy Farms in Mexico

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Summary: Animal welfare is of two basic arguments: ethical and productive. This paper evaluates dairy farms Holstein Friesian in an intensive system production. Farms are in Mexico State we evaluate: area of the pens, farmyard/animal, shade quantity per animal, watering hole space/animal, feeding and trough/animal, water quantity per animal besides it was realized a total injury count of necks and legs in animals. Data were analyzed and found that none of the farms has the proper amount of feed for each cow, the space required per cow is poor and the farms animals are injured, so no meets the 5 freedoms to assess the welfare of farm animals.

Introduction

Water supply in terms of quality and quantity is an important aspect from the point of view of welfare and productive. Bbacteriological quality of water has an effect on the milk quality and may cause mastitis, as dirty water can be found faecal coliforms, E. generally coli.

The heat stress associated with high humidity adversely affects milk production, along with a low reproductive efficiency. In contrast to excess shadow lack thereof increases the stress due to contact with sunlight, the animal rectal temperatures remaining in the sun increases considerably compared to those are protected with shadows and handled this shaded cows produced more milk with higher protein content than those who were under the sun.

Are two reasons that cows do not use the stall because space is swinging (stall width) is inadequate and the beds are uncomfortable. There is an association of the time when cows are lying comfortably and milk production. A study found that increases to 1.59 kg of milk per cow per day for each extra hour of rest received.

The coexistence of producing animals with other animals can be determined as a problem, for example since the close coexistence with cattle dog favors horizontal transmission of neosporosis.

It is important that people who are engaged in farming and live stock management awareness and understanding of their natural behavior, to facilitate their work and avoid accidents.

Material and methods

This research was carried out in 3 of the dairy farms in Mexico State. We evaluated the cow comfort in these stables (facilities management, health, nutrition, and behavior).

We used a questionnaire called "Score for cow comfort on the Dairy Farm", in order to assess that comfort and stables obtain stables that fell into the category of low comfort. Using records reproductive fertility was evaluated taking into account aspects such as calving interval calving, calving to conception, services per conception, conception dose, percentage of waste for reproductive problems, and age at first birth, these parameters were analyzed records card only primiparous cows breeding. Then calculated the percentage of all these aspects for the stable reproduction. Later data were sorted comfort and fertility data in SAS Institute JMP3.1.2 program in which there was a correlation statistically, comfort-fertility. With fertility data collected was descriptive statistics of the stables together. And finally there was a box where you mention the type of hormones that are used in each of the stables. Stalls with maximum comfort had an average value of fertility of 17.5 and those who obtained a minimum level of comfort was 8.63, the significance level was ($P = 0.13$), plus gave a $R^2 = 0.09$ indicating that fertility is explained in 09 to 1 by the comfort and 91 by other variables than the comfort, well if there is a positive relationship between the comfort found in cows and fertility of the same. Another result was that the stables evaluated only 14 stables working under conditions of cow comfort and the remaining 11 have minimum comfort conditions. Finally it is important to note that the services per conception; dose conception interval calving-calving, age at first birth, are slightly higher optimal values, but the values are not enough to cause problems, the percentage of waste for reproductive problems if it exceeds the value that indicates serious problems, and calving-conception interval is only if it falls within the optimum values based

on average this was obtained from the statistical.

Biweekly visits were made to each production in a period from May to July to observe and evaluate a questionnaire of 24 questions based on the five freedoms of animal welfare, of which six questions are directed to the owner of each ranch, these questions were intended to determine the type of food and the amount of feed per cow, milk production per day per herd, the number of dairy cows and the staff responsible for the animals. The other 18 questions are focused on observations and measurements of the site as well as the observation of the animal population. Measurements were made feeders, waterers, pens, sun decks, instead of grazing, amount of shade and stalls, in relation to the number of animals in each production. Besides measurements consider the type of material and quality of facilities and supplies that are given to cows. The questionnaire responses were processed in Excel tables, to make the necessary comparisons based on literature specifies Animal Welfare.

Results and discussion

In three farms evaluated observed that the feeding of dairy cows is not a function of the produce or the weight they have. The farms show a deficiency of 50% in the amount of food provided daily, which affects the welfare of the animal, as if the diet is not satisfactory and appropriate may be competition from cows during feeding, leading to aggression between animals.

Feeder spaces per animal of the three sites evaluated beyond standard measures mentioned (0.8 – 1.0 m) Los Angeles has a smaller extent (0.69 m) that standard (45 – 50 m) causing injuries and calluses on the neck to the nape of the neck. In two of the farms evaluated (El Escudo y Santa Rosa) is water ad libitum, while in Los Angeles have specific times where they are provided this vital, this practice increases animal stress by not having a source of that allows water to quench their thirst, at the three sites the water was dirty and in two of them had mold drinkers (Santa Rosa, El Escudo), according to Britter (2003), Drinkers measurements were performed to determine the amount of water per animal and per cow linear space taking into account that 10% of the animals are drinking water, according to literature. No operation has enough water for the number of animals that have, since the average water per animal per day should be 120 Lt.

Evaluations of pens in relation to the number of cows are greater than those reported by literature to recommend 8 to 12 m^2 per animal. The ratio of shade per animal must be of 15.24 m^2 one of the three farm holdings Farm El Escudo has deficiencies, while the Farm Santa Rosa and Los Angeles beyond the shadow space per animal, causing heat stress associated with moisture in the pen. Addition to having enough space in the farm, it must have a space for the cows can take a comfortable enough bed, on these site not exceeding the number of animals per stall, in the Santa Rosa farm and farm El Escudo they have enough stalls was observed that cows were selected to lie in one.

During injury assessments (foots and y neck), it was found that a minimum amount of cows suffering from this condition Green et al (2010) ensures that the hoof ailments are caused primarily by the type of bed and floor finish, it must have lined with anti-skid to prevent cows from slipping and hurting. These injuries cause distress due to pain and are a major problem in animal welfare. According to the evaluations conducted on two farms are present dogs are not trained and the Farm Los Angeles found that the dogs attack the cattle during grazing. Hemsworth et al. (2002) demonstrated as milk production increases when attitudes are improved staff and treatment of animals. Contrasted with the results and only the doctor in charge of each site are trained, while staff working all day with the animals.

Conclusions

None of the farms have knowledge of animal welfare, no farm meets the five freedoms. The staff working in each of the farms does not know the natural behavior of the cows; this makes them difficult handling them. The quality of water given to the animals is deficient in all three sites and the space required per cow feeder too.

The bunk space per cow in the 3 farms exceeds recommended by the literature cited. No clean feeders at Santa Rosa Farm and Farm el Escudo. The pen space per cow on farms exceeds the 3 recommended by the literature cited. The only operation that meets the recommended stall dimensions is the Farm Los Angeles.

In the Escudo Farm held of the amount of shade / cow is poor, while in the Santa Rosa farm there is excessive shade.

In the present study did not evaluate the effect of ventilation and lighting in milk production due to the lack of a methodology to help make these measurements.

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Epidemiology Investigation of Duck Viral Hepatitis in Some Areas of Shandong Province

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Summary: In order to understand the prevalence of duck virus hepatitis in Shandong, since August 2010 to December 2011, 31 suspected duck viral hepatitis samples have been collected from parts of Shandong and were studied on the incidence investigation, histopathological observation, virus isolation and identification, gene cloning and sequencing analysis. The results indicated that each region of Shandong has a high proportion of duck viral hepatitis cases, which mainly infected ducks under 4-week-old. Other days-age ducks can also be infected. Though cases were common in autumn, the seasonal feature of the disease is not obvious. It is different from a generally seasonal pattern of morbidity that has a peak in spring. The incidence and mortality of different duck flocks are diversity. The percent of paralysis has risen in clinical symptoms. Necropsy showed that, except characteristic lesions of viral hepatitis, some ducks had petechial hemorrhages on the endocardium and epicardium of the heart and some ducks had serious viral encephalitis lesions. 12 samples were DHV in the 31 samples. Among them, 11 samples were positive for DHV-Ⅰ (positive rate was 35.84%), 1 sample was positive for DHV-Ⅲ (positive rate was 3.23%). 17 samples were DHBV (positive rate was 54.84%). There were 4 samples, which were positive for DHV-Ⅰ and DHBV. The co-infection rate was 12.90%. Compared the gene sequences, mutant gene of DHV was less than DHBV. The survey shows that there is multiple hepatitis virus infection in Shandong duck flocks. Duck viral hepatitis incidence has increased. Symptoms and lesions of ill ducks appeared a few new features. These epidemiological changes are not only related to the changes in pathogenicity, may be also affected by appearance of duck TMU disease, mycotoxins and other drug poisoning.
Key words: duck viral hepatitis, epidemiological survey, pathological observation, isolation and identification of pathogen

Duck virus hepatitis (DVH) is a highly fatal contagious disease of ducklings, which mainly infects ducklings under 3-week-old. It is one of the major epidemics that seriously harm the duck industry. The three types in DWH are DHV-Ⅰ, DHV-Ⅱ and DHV-Ⅲ. The major clinical symptom is the special opisthotonus posture in death with hepatic hemorrhage, degeneration and necrosis. In order to know the prevalence of duck hepatitis in Shandong region, since August 2010 to December 2011, 31 suspected duck viral hepatitis samples were collected from parts of Shandong, and studied on the incidence investigation, pathological observation, virus isolation and identification, gene cloning and sequencing analysis, the detection of DHBV infected incidence. It provides theoretical foundations for the diagnosis and prevention of the duck disease.

Material and methods

Specimen

31 suspected duck viral hepatitis samples were collected from parts of Shandong, Taian, Laiwu, Zibo, Dezhou, Jining and Heze etc. The species of duck included egg-laying duck, beef duck, egg-laying breeding duck.

Epidemiology

Record the information, including breeding scale, management, species, clinical symptoms, prevention, therapy, morbidity, mortality, etc.

Pathology

Necropsy lesions External inspection and necropsy is done to observe the system organ lesions.

Microscopic lesions Fixing the liver, spleen, kidney, heart, lung, trachea, small intestine, glandular stomach, pancreas, brain tissue in 10% formalin solution, followed by routine paraffin section, HE staining for observing histological lesions.

Isolation and identification of pathogen

Chicken embryo incubation The liver and spleen samples were collected and homogenized with sterile 0.01 M phosphate-buffered saline (PBS with antibiotics (penicillin 10,000 IU/ml and streptomycin 10,000 IU/ml), pH 7.2). After freezing and thawing for three times, the homogenate was centrifuged for 20 min at 10000 *g*. The supernatant was inoculated into 11-day-old embryonic chicken eggs through the allantoic route, 0.2 ml per egg. The negative control was inoculated with NaCl solution. Embryonic chicken eggs were incubated at 37℃ for up to 96 h, giving up the dead embryonic chicken eggs in 24 h and then allantoic liquid of dead embryos was collected, storing −80℃ until use. Meanwhile subculture the allantoic liquid containing virus into the embryonic eggs five times.

Primer design and synthesis According to the DVH sequence in GeneBank, four pairs of primers were designed for PCR (Table 1), to detect DHV-Ⅰ, DHV-Ⅲ, Duck astrovirus, Duck Hepatitis B Virus. Primers were synthesized by Shanghai Sangon.

Table 1 Sequence of primers used in amplification of DHV gene

Gene	Primer	Sequence	Length/bp
DHV-Ⅰ	DHV1-F	GCTCCAGACTAGTTCCTGAGG	684
	DHV1-R	CGGAGATCCAAGATGGCATA	
DHV-Ⅲ	DHV3-F	CTAGGAGGTGGTGCTGAAA	254
	DHV3-R	CCCTCAGGAACTAGTCTGGA	
Duck Astrovirus	DastV-F	GGGAGGAGAGGCGTGACGGT	263
	DastV-R	TGCTGCACCCGCATCCTGTG	
Duck HBV	DHBV-F	CCCTTCACCCCAACGTGCGG	682
	DHBV-R	ATCGTCTTCCCGCCCCAGCA	

RNA extraction 250 μl allantoic fluid was collected from embryos which had significant lesions. The RNA was extracted on the basis of instruction books of Trizol.

DNA extraction 250 μl allantoic fluid was collected from embryos with significant lesions. The RNA was extracted on the basis of instruction books of DNAzol REAGENT.

RT-PCR A two-step method for acquiring cDNA from target gene was developed. Reverse transcription was in 25 μl system. PCR was in 60 μl system according to a conventional method.

Sequences of PCR outcome The PCR product was inserted into pMD18-T vector and transformed into DH5α cells. The bacteria solutions of selected positive recombinant were sent to Shanghai Sangon for sequencing. The sequence result was compared with that of the completed gene found in GenBank DHV nucleotide database.

Results

The incidence investigation

The findings show that, in recent years, amounts of DHV suspected specimen occur with the rapid development of ducks industry of Shandong. The disease has no obvious seasonality, but the higher incidence and the morbidity of the flock of duck appeared in autumn. Moreover, with Flavivirus prevalent in the past several years, the incidence of the suspicious DHV accompany had increased. The survey results indicated that there was a distinctive difference in the morbidity and mortality at duck farms of different regions.

Clinical symptoms and post-mortem

The mainly manifestations of 31 suspected samples are pyrexia, anorexia, depression, higher paralysis, the backstroke shape, no obvious respiratory symptoms. The symptoms of sick ducks include swing dropping, depression and the dead with the opisthotonus posture.

The gross lesion in the sick duckling liver is multiple mottled hemorrhage (Fig. 1B, C); the greater duck liver is swollen, discoloration or khaki, fragile, even the fatty degeneration (Fig. 1D); cellulose hepatic pericarditis for secondary infection; Gallbladder swollen with full of green bile and grayish white necrotic foci of the spleen (Fig. 1E), some with kermesinus or mottled hemorrhagic necrosis; some with petechial hemorrhage on the endocardium of the heart (Fig. 1F).

Microscopic lesions

Severe hemorrhagic necrosis of duckling liver (Fig. 2A), many chronic cases has extensive bile duct hyperplasia, various levels lymphocyte infiltration and hemorrhage (Fig. 2B, C), lighter liver cells with particle degeneration and blisters degeneration, heaver diffusively fatty degeneration and necrosis (Fig. 2D); Spleen presents various levels necrotic lesions, lymphocytic necrosis and disintegration (Fig. 2E); Renal tubular epithelial cells showed general granular degeneration or blisters degeneration, interstitial congestion, lymphocyte proliferation and mild viral encephalitis (Fig. 2F).

Detection of virology

PCR detection 11 of 31 samples are DHV-Ⅰ positive (Fig. 3A). The positive rate was 35.48%; astrovirus was negative; 1 DHV-Ⅲ was positive (Fig. 3B), the positive rate was 3.23%; 17 DHBV were positive, the positive rate was 54.84%. Among them, there were four samples with DHV-Ⅰ and DHBV double positive, the co-infection rate was 12.90%.

PCR sequence determination and analysis

Determination and analysis of DHV-Ⅰ VP0 gene sequence: The isolated virus was more than 90% identical with the counterpart of DHV-Ⅰ on the NCBI, the results showed that the isolated strain was DHV-Ⅰ.

The VP0 gene of 5 DHV positive samples (Numbers 12, 49, 78, 89, 92) were sequenced, showing that the different strains shared 93.8% – 100% homology at the VP0 gene. 70.4% – 97.5% identical with other reference strain. The isolated strain 89 and 92, belonging to the same strain isolated from different samples, shared 100% homology at the nucleotide level. 49 and DHV1-

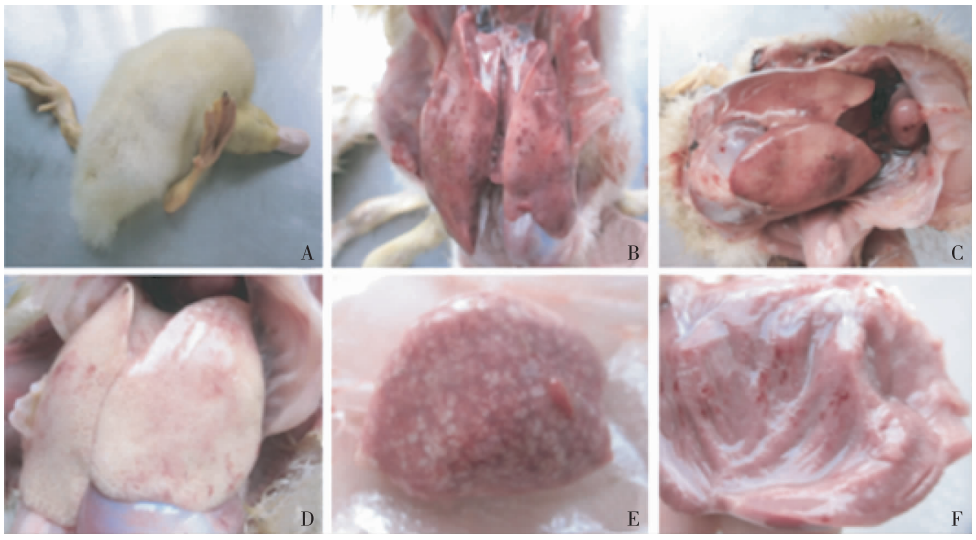

Fig. 1 Clinical symptoms and necropsy lesions of suspected duck viral hepatitis. A. Dead duck was opisthotonus posture; B, C. Liver spotted hemorrhage; D. Fatty degeneration of the liver; E. Grayish white necrotic foci of the spleen; F. Petechial hemorrhages on the endocardium of the heart.

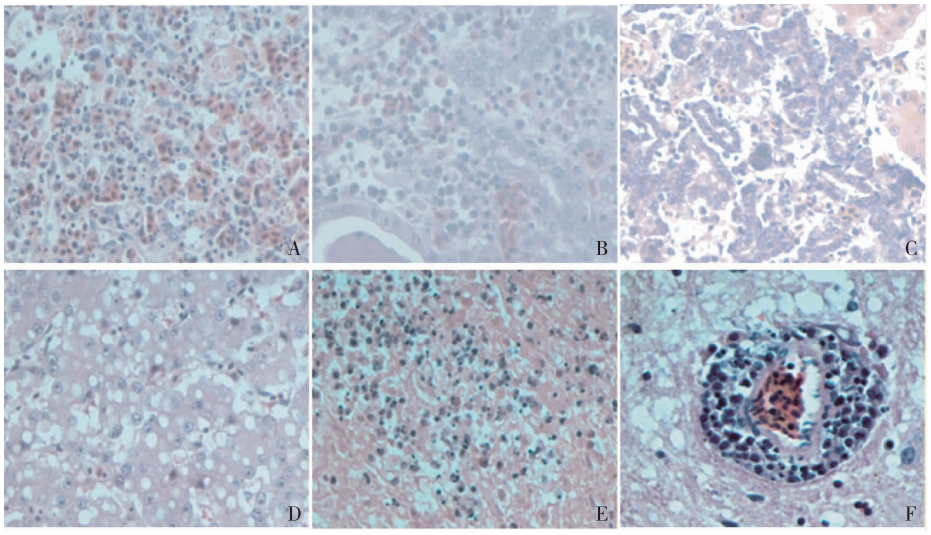

Fig. 2 Histopathological changes of suspected duck viral hepatitis. A. Hemorrhage, necrosis of liver, 400 ×; B, C. Hemorrhagic necrosis of the liver, bile duct proliferation, lymphocyte proliferation, 200 ×; D. Fatty degeneration and necrosis of liver, 400 ×; E. Necrosis and disintegration in splenic lymphocytes, 400 ×; F. Lesions of virus encephalitis, 400 ×

03D reference strains had the higher homology, 97.5%; 12 and DHV1 - YZ reference strains homology, 95.6%; 78 and ZJ reference strains homology, 97.2%; DHV1 89, 89 and DHV1-03D reference strains homology, 97.4% (Table 2, Fig. 4).

Determination and analysis of DHV-Ⅲ gene sequence: The isolated virus was over 90% identical with the counterpart of DHV-Ⅲ on the NCBI, the results showed that the isolated strain was DHV-Ⅲ.

The 5 'UTR gene of DHV positive samples (number 24) was sequenced. Comparative genome analysis with other available reference strains indicated that JX strain shared 60.9% – 100% similarity at the nucleotide level, the higher similarity with the DHV-Ⅲ sequence, the higher homology with C-YDF reference strain, the lower similarity with the DHV-Ⅰ sequence.

Discussion

According to the incidence investigation of the suspected DHV samples from different regions in Shandong province, in recent years, with the rapid development of ducks- raising industry, DHV suspected specimen increased. In the past, it mainly occurred in spring, but now, it has no obvious seasonality, happens all over the

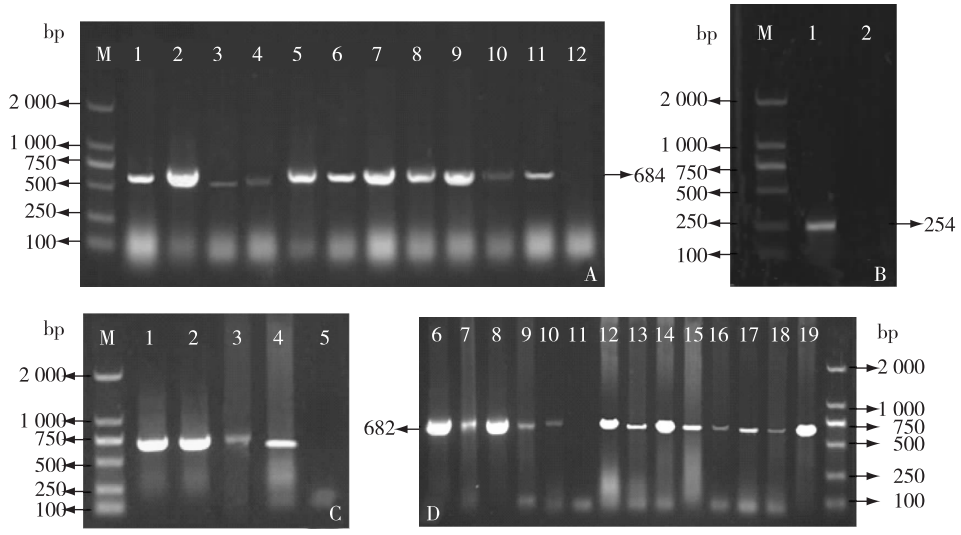

Fig. 3 A. The PCR results of DHV-Ⅰ of tested ducks(1 – 11. Detected samples; 12. Negative control); B. The PCR results of DHV-Ⅲ of tested ducks(1. Detected samples; 2. Negative control); C, D. The PCR results of DHBV of tested ducks(1 – 10, 12 – 19. Detected samples; 11. Negative control; M. DL2000 marker)

Table 2 Similarity analysis of nucleotide sequences of VP0

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		
1		95.3	94.5	95.4	95.3	95.3	94.7	94.0	95.0	94.7	73.3	73.5	73.4	73.3	73.0	72.9	95.4	94.3	95.1	94.9	95.2	95.2	1	DHV1-JX
2	5.0		95.1	96.5	95.9	95.8	97.2	95.1	95.8	95.6	73.3	73.4	73.4	73.3	73.2	73.2	96.0	94.7	97.5	96.1	97.4	97.4	2	DHV1-03D
3	5.8	5.1		94.8	96.2	96.0	94.4	95.0	96.2	95.0	73.4	73.4	73.4	73.4	73.0	72.9	98.7	95.6	95.1	94.6	94.9	94.9	3	DHV1-YZ
4	4.8	3.6	5.4		95.8	95.7	95.8	94.5	95.4	95.3	73.4	73.3	73.4	73.5	73.0	72.9	95.8	94.4	96.6	95.1	96.4	96.4	4	DHV1-C80
5	4.9	4.3	3.9	4.3		99.1	95.2	95.5	97.0	95.6	73.5	73.4	73.5	73.6	73.4	73.4	97.1	93.3	94.7	94.3	95.2	95.2	5	DHV1-R
6	4.9	4.3	4.2	4.5	0.9		95.1	95.4	96.6	95.5	73.5	73.5	73.5	73.5	73.4	73.3	96.9	93.4	94.9	94.4	95.4	95.4	6	SG
7	5.6	2.8	5.9	4.4	5.1	5.1		94.2	94.9	94.8	73.4	73.4	73.4	73.5	72.9	72.9	95.2	94.9	97.0	97.2	96.2	96.2	7	ZJ
8	6.4	5.2	5.2	5.8	4.6	4.8	6.1		95.7	94.5	73.0	73.2	73.3	73.1	73.0	73.0	95.9	93.8	95.6	95.1	95.4	95.4	8	5886
9	5.3	4.4	3.9	4.7	3.1	3.5	5.3	4.4		95.4	73.5	73.5	73.6	73.6	73.2	73.1	97.1	94.3	95.7	94.6	96.1	96.1	9	DRL-62
10	5.5	4.6	5.2	4.9	4.6	4.7	5.5	5.7	4.8		73.5	73.6	73.5	73.4	73.4	73.3	95.8	93.4	93.4	93.4	94.1	94.1	10	DHV1-H
11	34.2	34.0	33.9	34.3	33.8	34.0	34.2	34.5	33.9	33.8		99.1	98.6	99.3	78.6	78.5	73.4	74.9	75.2	74.2	75.2	75.2	11	AP-04009
12	34.0	36.9	33.8	34.1	33.7	33.9	34.1	34.4	33.8	33.8	0.9		98.7	99.3	78.6	78.6	73.4	75.2	76.0	75.0	74.9	74.9	12	AP-04114
13	34.1	34.0	33.8	34.2	33.7	33.9	34.2	34.3	33.8	33.8	1.4	1.3		98.8	78.5	78.5	73.4	74.4	73.6	72.6	73.6	73.6	13	AP-04203
14	34.2	34.0	33.7	34.1	33.7	33.9	34.1	34.4	33.7	33.7	0.7	0.7	1.2		78.5	78.4	73.4	74.9	75.2	74.2	75.2	75.2	14	AP-03337
15	35.0	34.6	34.7	35.0	34.4	34.4	35.1	35.0	34.4	34.4	25.7	25.6	25.7	25.8		99.6	73.0	72.2	70.6	70.6	71.1	71.1	15	90D
16	35.2	34.7	34.8	35.0	34.5	34.5	35.1	35.1	34.6	34.5	25.7	25.7	25.8	25.9	0.4		73.0	72.4	70.8	70.7	71.3	71.3	16	04G
17	4.8	4.1	1.3	4.4	2.9	3.2	5.0	4.3	3.0	4.4	34.0	33.8	33.9	33.8	34.7	34.8		95.1	95.6	94.4	95.4	95.4	17	R85952
18	6.1	5.5	4.6	5.9	7.2	7.0	5.3	6.6	6.1	7.0	32.1	32.1	32.9	32.1	35.2	35.5	5.2		94.3	93.8	94.7	94.7	18	12
19	5.5	2.9	5.5	3.9	5.9	5.7	3.4	5.0	4.8	7.4	31.0	30.5	31.8	31.0	38.2	38.4	5.0	6.1		96.2	96.6	96.6	19	49
20	5.7	4.4	6.1	5.5	6.4	6.2	3.2	5.5	6.1	7.4	32.6	32.1	33.4	32.6	38.4	38.7	6.3	6.6	3.9		95.1	95.1	20	78
21	5.3	3.0	5.7	4.1	5.3	5.1	4.3	5.1	4.4	6.6	31.8	32.3	32.6	31.8	37.9	31.8	5.1	5.5	3.6	5.1		100.0	21	89
22	5.3	3.0	5.7	4.1	5.3	5.1	4.3	5.1	4.4	6.6	31.8	32.3	32.6	31.8	37.9	38.1	5.1	5.5	3.6	5.1	0.0		22	92
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		

year and has more cases in autumn. The morbidity of the flock of duck had distinction, related to infected pathogen, age, level of maternal antibody, immunity, management, co-infection. In recent years, the occurrence of Flavivirus, the prevalence of aflatoxicosis and toxics of therapeutic drug greatly promote DHV epidemics and obviously make the diagnosis of DHV difficulty. Just as aflatoxicosis and Flavivirus, the worst season of the DHV incidence is also in autumn. Compared with the past condition, DHV has more serious symptoms of paralysis, endocardial hemorrhage, obvious viral encephalitis, no significant effect of high immune serum injection in some cases. The emergence of the abnormal conditions indicated that some cases were related to the Flavivirus infection or aflatoxin and toxics of other drug. My partuers in the laboratory have already proved that the flock of sick ducks has the higher percent co-infection of Flavivirus and the problem of Aflatoxin overdose.

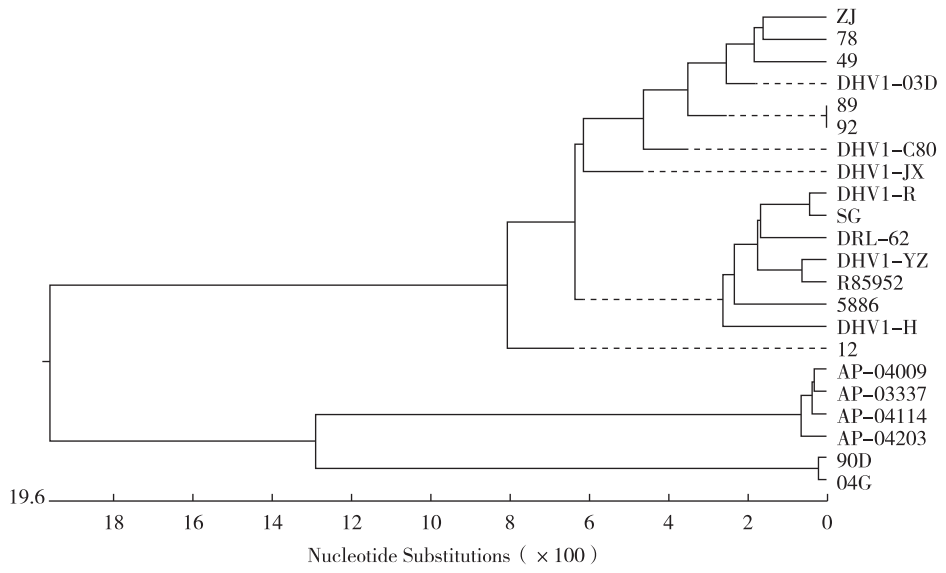

Fig. 4 Phylogenetic tree of the VP0 gene for DHV-Ⅰ strains

PCR detection results showed that the mainly prevalent DHV in duck was DHV-Ⅰ, respectively DHV-Ⅲ, no astrovirus (DHV-Ⅱ); DHBV also had higher positive rate. This result showed the complexity of the DHV epidemiology, especially with higher percent DHBV positive and co-infection. Although the DHBV was negative infection to duck, we should put emphasis on the potential threat to human healthy and safety problems. There were 3 DHV-Ⅰ positive samples for egg-laying duck or breeding duck. Y. m. Saif reported that adult duck could be infected but no clinical symptoms and no influence of the egg-laying rate. Serum and egg contained neutralization antibodies[1 - 6]. But this study found that DHV-Ⅰ existed in the egg-laying duck liver and liver had degenerative lesions. Therefore DHV-Ⅰ may pose threat to the egg-laying duck. Adult duck infection played a significant role in the spread of the disease. This condition should cause the attention of people.

There were no finding haemagglutination virus by hemagglutination test in the chicken embryo allantoic fluid of 31 samples incubated, without the infection of avian influenza and paramyxovirus. By comprehensive analysis, suspected DHV detection rate was only 38.7%, the misdiagnosis rate was as higher as 60%. Therefore, there were some difficulties in the DHV diagnosis, only dependent on the symptoms, post-mortem and histopathology diagnosis, especially with the misdiagnosis of Flavivirus or aflatoxin and toxics of other drug. Virology test diagnosis was the essential process.

The identity of DHV-Ⅰ positive samples VP0 gene with the reference strain at the nucleotide sequences level was 93.0% - 97.5%. Although there were individual base mutations, whole mutation was no distinction. Each isolated strains with Taiwan DHV1-03D reference strains homology was higher. DHV -Ⅲ positive samples 5' UTR gene shared 96.5% - 100% homology with reference strains, 100% identical with C-YDF reference strain. The finding showed that 5' UTR conservative sequences had no mutations. Due to no distinctive mutations in the DHV separation strain gene sequences[7 - 9], injecting high immune serum especially containing Ⅰ type and Ⅲ type of serum into the sick duck should receive a good treatment effect, unless there was the co-infection of other pathogens.

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Can Animal Protein Rich Diet Reduce the Frequency of Injuries in Beak Trimmed and not Beak Trimmed Turkey Hens?

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Summary: The origin of feather pecking and cannibalism in turkeys is still largely unknown. The present practice of beak trimming to minimise injuries in the pecked birds will probably be banned by 2018 in Lower Saxony because of welfare reasons. This study investigates whether an animal protein rich diet can reduce feather pecking and cannibalism in female fattening turkeys (B. U. T. 6). A total of 5080 turkeys were split in four groups of 1270 birds each and kept in a commercial barn during two fattening periods on woodchips (up to 6 weeks of age) and later on straw litter. The beaks of the turkeys in two of these groups were trimmed whilst the beaks of the turkeys in the other two groups were not treated. One group of beak trimmed turkeys and one group of birds with intact beaks were provided with a diet that contained animal protein (hemoglobin powder and fish meal). The two other groups were fed with a plant-based diet as usual. Twice a day the animals injured birds were separated from the groups. Pathological investigations were performed on all culled and found dead turkeys with special attention given to external injuries. The results showed that between 3. 8% and 6. 4% of the hens in the groups with untreated beaks had to be separated during the two fatting periods because of injuries while between 0. 8% and 2. 9% of the animals of the groups with trimmed beaks had to be taken out of the flock. There were three times (second trail) and four times (first trail) more injured turkeys in the groups of hens with intact beaks than in the groups with trimmed beaks. This orientating investigation shows that at least 0. 47% (6 animals) and up to 1. 80% (23 animals), nearly twice as many turkeys died of massive injuries (cannibalism) in the groups of animals with intact beaks than in the groups of turkeys with trimmed beaks (0. 23% -3 animals to 0. 63% -8 animals). It appears that the animal protein diet does not have an effect on the intensity of feather pecking, cannibalism and resulting injuries in this investigation.

Introduction

In 2011 more than 35 million turkeys were raised in Germany (Destatis 2012). Nearly all turkeys were beak trimmed in order to minimize the effects of feather pecking and cannibalism. It is planned to ban beak trimming from 2018 in Lower Saxony but experiences with measures how to prevent feather pecking without beak trimming are still scarce.

Feather pecking (FP) and cannibalism (C) are well known behavioural disorders which can cause poor welfare in the birds and considerable economic losses in turkey production. Aggressive pecking on the head of their flockmates is observed in wild turkeys as well as in domestic turkeys (MOINARD et al. 2001). Some authors argue that FP and C are induced by the poor environment in the keeping systems of the modern fattening turkey (SCHLUP et al. 1990; MARTRENCHAR 1999). Injuries caused by pecking can lead to reduced growth or death or death by culling (Huges and Grigor 1996; Sherwin et al. 1998). To minimize the negative effects of this abnormal behaviour the birds are routinely beak trimmed at early age. But even this intervention does not solve the problem completely. In Germany, it was observed that 12. 8% of the male and 13. 8% of the female beak trimmed turkeys in conventional production had injuries at the age of 16 weeks (KRAUTWALD-JUNGHANNS 2012). 9. 42% of the injuries were caused by penmates and the snood was the most frequently injured region. Neither beak trimming is desirable from the perspective of the welfare of the turkeys nor do these practices improve the public image of intensive farming systems. Furthermore, the government of Lower Saxony has recommended to stop beak trimming as soon as possible, latest in 2018. Therefore there is enormous interest in finding a strategie which can help to reduce FP and C.

Although the turkey is since long an important commercial species there is very little known about the causes of FP and C. The aetiology of these behavioural disorders seems to be a complex interaction of different factors. Several authors have discussed genetics influences, environmental factors like not suitable indoor climate or light regime, lack of environmental enrichment structures, social stress caused by group size and stocking density but no clear solution was drawn (SCHLUP et al. 1990; CROWE and FORBES 1999; SHERWIN et al. 1999; MARTRENCHAR 1999; PETERMANN and FIEDLER 1999; MARTRENCHAR et al. 2001; BERK 2002; BERK and HINZ 2002; BUCHWALDER and

HUBER-EICHER 2004; FIEDLER and K NIG 2006). One effect on the incidence of feather pecking and cannibalism is also attributed to the composition of the feed. It is known that wild turkeys eat insects, spiders, small invertebrates and grasshoppers (SCHORGER 1966) while the diet for commercial fattening turkeys does not contain animal protein. Therefore, this study set out to focus on the possibility that the inclusion of animal protein in the diet decrease the frequency of pecking injuries.

Material and methods

Two consecutive trails were conducted on turkeys for a period of 16 weeks. A total of 5080, day-old, female turkey poults (B. U. T. 6) were used in each fattening period. These birds were allotted to four groups and fattened under practical conditions in a confined building with four identical barns, each with a floor space of 238 m^2.

The beaks of the turkeys in two groups were trimmed whilst the beaks of the turkeys in the other two groups were not treated. One group of beak trimmed turkeys and one group of birds with intact beaks were provided with a diet containing animal protein. The two other groups were fed a common plant-based diet. The diet was subdivided into five different phases depending on the weight of the turkeys. As animal protein source between 2% and 5% fish meal and between 2.8% and 3.07% hemoglobin powder of pigs were used.

The daily animal inspection was done two times a day, one between 8 – 9 a. m. and the other one 3 – 4 p. m.. Any sick or injured birds that required attention were separated from the group. The number of injured birds, injured body regions and date of the injury were recorded daily. After recovering the hens were set back to their flock. Pathological investigations were performed on all culled and found dead turkeys with special attention given to external injuries caused by pecking.

Results and discussion

Table 1 shows the percentage (in %) and number of separated hens in % frequency (in %) and the cumulative number of separated hens with injuries and the number of turkeys died and culled by cannibalism in the four groups during the two fattening periods (16 weeks). In the groups with untreated beaks and conventional diet 3.8% and 6.4% of the birds had to be separated because of wounds during the two trails. In contrast, only 0.8% and 2.9% of the animals of the groups with trimmed beaks and conventional diet had to be taken out of the flock because of injuries, this differs by a factor three and four, respectively. In the groups with untreated beaks and animal rich protein feeding a total of 56 hens (4.4%) during the first trail and 76 birds (6.0%) during the second trail had to be separated because of skin lesions which are almost the same numbers as in group A (beak not treated and conventional diet). This indicates that the diet with animal protein does not have an effect on the frequency of pecking injuries in this study. The results of the pathological investigations show that the number of turkeys killed by pecking damage was overall much higher (> two-fold) in the groups of not beak trimmed hens than in the two groups of treated hens. At least 0.47% (6 animals) up to 1.80% (23 animals) turkeys died or had to be culled because of pecking damage in the groups with intact beaks while 0.23% (3 animals) up to 0.63% (8 animals) in the groups of turkeys with trimmed beaks. When comparing the two different diets no influence of the animal protein on the incidence of birds killed by pecking lesions could be found. An equal number of birds (6 hens in the first and 23 animals in the second trail) were killed by injuries in the two groups of not trimmed hens regardless of the feeding. The presented data of this orientating investigation show that beak trimming but not the used animal rich diet had the potential to reduce injuries, cannibalism and feather pecking.

Table 1 Number and percentage (% of introduced hens) of hens separated because of injuries and turkeys died and culled by injuries in the four groups during each trail

Group	Treatment	Trail	Percentage and number of separated hens in %	Percentage and number of turkeys died and culled by injuries in %
A	not beak trimmed + conventional diet	1	3.8% (48 birds)	0.47% (6 birds)
		2	6.4% (82 birds)	1.80% (23 birds)
B	not beak trimmed + diet with animal protein	1	4.4% (56 birds)	0.47% (6 birds)
		2	6.0% (76 birds)	1.80% (23 birds)
C	beak trimmed + conventional diet	1	0.8% (10 birds)	0.31% (4 birds)
		2	2.9% (28 birds)	0.63% (8 birds)
D	beak trimmed + diet with animal protein	1	1.3% (16 birds)	0.23% (3 birds)
		2	1.7% (22 birds)	0.47% (6 birds)

Conclusions

1. The results indicate that there is a cannibalism problem also in groups of beak trimmed turkeys.

2. Beak trimming did not entirely prevent that turkeys were killed by pecking injuries.

3. The incidence of turkeys with pecking injuries was three to four times higher in the groups with not beak trimmed birds compared to the groups with trimmed beaks.

4. 23 birds (1.8%) each of the initially housed turkeys were killed by flock mates (cannibalism) or had to be culled because of injuries in the two groups of animals with intact beaks, compared to 0.23% to 0.63% in the groups with beak trimmed hens.

5. In this investigation the use of diet with animal protein did not display any visible effect on the number of injuries and cannibalised birds.

6. It is necessary to put more research efforts in this complex and multifaceted problem of feather pecking and cannibalism.

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Stray Animals and Public Health

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Summary: Stray animals are home animals (or companion animals or pets) which were abandoned by people or escaped from home, mainly refers to the cats and the dogs. People abandoned stray animals for many reasons, the likelihood is urban demolition, family change, animals illenss, being tired of the pets in affection. Deeper reason might be: the lack of relating knowledge of raising pets, the lack of relevant law enforcement and the lack of animal ethics.

As the number of pets increases day by day, the number of stray animals leaps. China has almost 100 million dogs, of which about 10% are the stray dogs. Stray animals proliferation, brought a series of public health problems, such as spreading disease, creating noise and environmental pollution, causing traffic accidents and so on. Recently, those problems have aroused the concern of the whole society. In order to improve the urban public health level and the stray animal welfare, humane control of stray animals population can be carried out by strengthening the animal protection, spreading the idea of animal welfare, educating veterinarian students, improving citizens sense of responsibility. Also, the number of stray animals can be reduced by using the methods like sheltering, neutering and immuning, thereby improving the whole social public health level.

Introduction

Dogs and cats family animals (pets) are domestic animals. If they are abandoned or escape from home and gradually live independently without relying on human, those animals will be called the rewilding domestic animals, namely stray animals. With the growing number of urban rearing pets, abandoned domestic animals appear. After being abandoned, some animals died silently, while others survived tenaciously in cities, becoming stray animals that can be seen everywhere. In all those stray animals, the most concerned abandoned animals are dogs and cats, the spread of which may cause a series of public health problems. Paying attention to the stray animal welfare and survive is significant to human health protection and public health security.

Results and discussion

The cause of stray animals

Following phenomena are common in China. First, houses dismantlement in city. Thousands of cats and dogs were abandoned with the old houses dismantled in city. According to the investigation of the *International Fund for Animal Welfare* (*IFAW*), nearly 90% of households of the dismantled buildings wouldn't take the dogs away with them after house dismantled. Secondly, family changes. Many cats and dogs became the victim of family divorce or disputes. Third, the animals get sick: Many pet owners are not willing to pay for the treatment of sick pets. Fourth, some owners of pet kept the pet just for fun and playing. When they lose interest and tired of playing, they throw away the pets.

Several problems can be found by detail analysis the above phenomena.

First, Lack of the knowledge of pets feeding. Many owners of pets took the pet home without seriously thinking and adequate preparation. They usually lack the knowledge of pets feeding. Such as, the enough space, time, economy are needed for pet feeding; knowledge of feeding, management and disciplining are also needed. Many owners get into troubles to feed pet as lacking the knowledge of pets feeding. As a result, some of them choose to abandon the pets and let them wandering everywhere. These would cause serious problem.

Secondly, lack of relevant law for enforcement. Currently, there are no laws to prohibit torture of animals, abandon of pets, selling of cats and dogs and other improper behaviors in China.

Third, the lack of ethics and moral. The proportion of people keep pets increased in China, while the phenomena of pets abandoned were also increased at the same time. Stray animals which caused by lacking of the ethics and moral in the treatment of animals and lacking of the corresponding rules to guarantee the animal welfare have became the common social problem in many cities. The topic of love animals caused a wide range of discussion and social attention after cases of the extreme cruelty to animals. Animal abuse and abandon is trampled life behavior, and reflects the lack of social morality, the social progress and the level of civilization.

Thus, the behavior of animal abuse and abandon should be forbidden.

Welfare of stray animals

Animal welfare means how an animal is coping with the conditions in which it lives. It was firstly put forward in the farm animals, and then applied to almost all animals, such as laboratory animals, companion animals, wild animals and aquatic animals, etc. The standard and core of the animal welfare evaluation is five freedoms, including freedom from hunger, thirst and malnutrition; freedom from fear and distress; freedom from physical and thermal discomfort; freedom from pain, injury and disease; and freedom to express normal patterns of behavior. Due to lack of people's love, the welfare of stray animals has been very poor, mainly displays in the following phenomenas.

a. Stray animals often suffer from hunger and thirst, so they have to seek for food and dwellings in the communities of people living.

b. Stray animals often suffer from parasitic diseases and infectious diseases, such as the skin diseases caused by fleas, ticks and mites.

c. In order to control the number of stray animals, developing countries often choose to kill the stray dogs with poisoning, electric shocking or shotting at will. These inhuman methods cause the animals great pain and suffering.

e. Stray animals are always threatened, kicked, shocked and even abused by strangers.

f. The competion for spouse (or mate) and food among stray animals may lead to fighting, therefore cause trauma and nervous.

g. Stray animals are often captured by unscrupulous businessmen and sold to markets and restaurants. These dogs and cats are eaten by decorticating alive which strongly infects the animal welfare.

Public health problem caused by stray animals

Spread the disease:

Stray animals are host of a lot of diseases infected human beings and livestock, such as rabies (rabies), Echinococcus disease (Echinococcus) and bow ascariasis (*Toxocara Canis*), etc. At present, China is one of the high incidence areas of rabies in the world, the epidemic situation of which is going severe year by year. Every year, the number of patients died because of rabies is about 2000, second-biggest in the world that only after India. The main reason of the problems is the rising number of pets in both urban and rural areas, and of stray animals.

The direct physical injuries toward humans and other pets:

Stray dogs apparently may bite pedestrians, pets or other animals leading to physical injuries or infection with rabies. They may also chase livestock (such as sheep) that can cause abortion and trauma, resulting in great economic loss.

Causing traffic accidents:

Since stray dogs and cats ran around the city streets, elevated and high speed highways, car drivers may turn suddenly to avoid killing them. The accidents then happen as a result, causing severe injuries and even death. According to news reports, the growing population of stray dogs and cats is the hidden danger that causes more than 10 traffic accidents one year.

Causing environmental pollution:

Stray animals often rummage garbage bags and trash cans to find food, then drop garbage everywhere, making second waste pollution. At the same time, stray animals defecates their excrement and urine anywhere, which also cause environmental pollution.

In the breeding season, barking from stray cats and fighting among stray dogs make noises that lead to sound pollution. Meanwhile, the aggressive behaviour is annoying and threatens people's safety and health.

How to control stray animals

The world organization for animal health (OIE) in the Animal Welfare part of *Terrestrial Animal Health Code* introduces the stray dog group control, formulates the dog group control plan goals, including: improve health and welfare of owned and stray dog population; reduce numbers of stray dogs to an acceptable level; promote responsible ownership; assist in the creation and maintenance of a rabies immune or rabies free dog population; reduce the risk of zoonotic diseases other than rabies; manage other risks to human health (e. g. parasites); manage other risks to human health (e. g. parasites); prevent harm to the environment and other animals; prevent illegal trade and trafficking.

As for China, controlling the stray animals shall be conducted mainly on the following aspects:

a. To strengthen legislation. National People's Congress shall enact laws and policies like the *Animal Protection Law of PRC*, *Cruelty to Animals ACT* in order to regulate people's action as soon as possible.

b. To strengthen the public education. The ideas of animal protection and ethics shall be introduced to the masses. In the process of pet-raising, owners' sense of responsibility shall be improved that neither abandon nor abuse to pet will be allowed. If one can't be responsible to his pet, the pet shall be sent to the standard animal shelter or related institutions.

c. To establish strays care institutions, such as all kinds of different forms of small animal protection

associations. Non-governmental individuals and NGOs shall be encouraged to participate themselves in operating stray animal asylums. According to the data from Chinese Aademy of Social Sciences, there are more than 10,000 non-governmental animal rescue organizations. Among them, about 2,000 began to take shape. They are spread the importance of helping animals to more people, delivering warmth to animals in need. For example, Nanjing Peace ArFu stray animal shelter is taking care of at least 1,800 stray animals.

d. To strengthen neuter. In order to curb stray animal growth rate, desex surgery is widely used worldly as an efficient method. In recent years, under the coordination of governmental departments, each animal protection societies, related institutions of higher education and scientific research institutions have been involved in a stray cat "Neuter" work. Currently, the most effective accepted means to control stray cats population is "TNR", namely "Trap"-"Neuter"-"Release".

e. To strengthen veterinary education. Veterinarian students shall be trained with the concepts of animal protection while the teaching content of animal welfare shall also be enhanced. Educators shall have the responsibility to ensure that students after graduation will operate ethically in jobs and will not perform operations like docking, cropping or vocal cord cutting that impair animal welfare.

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Identification of Biofilm Formation by *Mycoplasma gallisepticum*

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Abstract: *Mycoplasma gallisepticum* is the causative agent of chronic respiratory disease in chickens and of infectious sinusitis in turkeys, chickens, game birds, pigeons, and passerine birds of all ages. This study investigated the biofilm-producing ability of *M. gallisepticum* strains in an attempt to explain its intriguing persistence in commercial flocks. Eleven strains of *M. gallisepticum* were investigated for their biofilm formation, which varied considerably. Strains Nobilis MG 6/85, S6 (P5 and P20), D9604, and SU15 were strong biofilm producers. Strains Rlow (P10 and P100), NCL, CG5, YL4, and F were weak biofilm producers. Strains Vaxsafe MG ts-11 and F36 did not produce biofilm as verified using a crystal violet staining assay. In addition, highly differentiated biofilm structures of strain Nobilis MG 6/85 with characteristic stacks and channels were observed under confocal scanning laser microscopy and scanning electron microscopy. The carbohydrates (sucrose, glucose), disodium ethylenediaminetetraacetic acid (EDTA), antibiotics (tetracycline, gentamicin), or detergent (Triton X-100) were further used to determine their effects on biofilm formation. Biofilm formation was significantly inhibited by 5% sucrose and 5 mmol/L EDTA. Compared with the planktonic mycoplasma, these biofilm-grown cultures were more resistant to tetracycline, gentamicin, and Triton X-100 treatments. Furthermore, real-time reverse transcriptase-polymerase chain reaction was performed to investigate the transcription of several genes that may be associated with biofilm formation. The results indicated that the transcriptions of some genes in the biofilm-grown cells were markedly decreased, including vlhA3. 03, csmC, hatA, gapA, neuraminidase, and mgc2. Our results will benefit further research on the persistence of *M. gallisepticum* infections.
Key words: *Mycoplasma gallisepticum*, biofilm, formation, biofilm variation

Applying the Welfare Quality Protocol in the Assessment of Dairy Cow Welfare in Different Housing Systems

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Abstract: The aim of this study was to assess the welfare of dairy cows raised in tied and free stalls from selected dairy farms in South-Western Sweden. A questionnaire for dairy cows welfare assessment was set up using the Welfare Quality® protocol, as well as following discussions with researchers and specialists in this area. A total of 20 farms were assessed, and the animal-based measures were evaluated on 35 cows in each farm. Data were statistically processed using Statistica software, and differences between the two housing systems were tested using chi-squared test (χ^2) or Mann-Whitney U test. At individual level, cows had cleaner tarsi and udders ($P < 0.001$), as well as the percentage of lame cows was lower ($P < 0.001$) when housed in tie stalls compared to free stalls. At farm level, the percentage of cows with integument swellings on the neck, shoulder and back was significantly higher in free stalls ($P < 0.05$), while the percentage of cows showing hairless areas on carpus was higher in tie stalls ($P < 0.05$). The housing system had no influence on the overall qualitative behaviour assessment score (19.46 in free stalls vs. 18.75 in tie stalls), although cows in tie stalls were calmer, more relaxed and bored compared to cows in free stalls that were more agitated. At the welfare criteria level, free stalls offered a higher movement freedom for cows, while cows in tie stalls had lower frequency of injuries and better relationship with man. Only the good housing principle was significantly influenced by the housing system, a higher score being obtained for free stalls compared to tie stalls (59.5 vs. 35.6, $P < 0.05$). The cow welfare in the visited farms were categorised as being enhanced or acceptable, with 60% of the free stalls and 30% of the tie stalls farms classified as enhanced.

The EEG Application as an Assessment Method of Animal Welfare

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Abstract: Welfare is defined as the state of physical and mental health of the animals which is reasonably achievable under conditions of complete harmony between the organism and the surrounding environment. The paper presents the electroencephalographic study (EEG) as useful diagnostic method of animal welfare evaluation. The EEG summarises the electrical activity generated by neurons in the cerebral cortex. This phenomenon is a specific tool used for receiving and recording bioelectrical activity from the central nervous system, as well as for analysing its basic functions. Most of the body's reactions take place in the brain, therefore monitoring of its functions, including the response to various stimuli, may be helpful in physiological and behavioural methods of animal welfare assessment. The long-term (24 h) analyses of bioelectrical brain activity were made simultaneously on clinically healthy rams with the use of ambulatory EEG Holter units. All of the data from the EEG studies were obtained in digital records. A comparative analysis was made taking into account free artefact samples of the EEG. The analyses of the animals' physiological state as HR/min. (84. 01 ± 6. 22), RR/min. (56. 89 ± 6. 47) or RT (38. 99 ± 0. 18) were also performed. It was found that the standard electroencephalographic records in sheep registered in wakeful and conscious states was a rhythmic activity within the range 5. 6 Hz. The amplitude of the EEG does not exceed 50 μV (mean 21 μV). The higher values do not constitute the existence of pathological features. The use of the EEG methods in sheep provide the estimation of the stressful factors on the organism and determine the level of animal welfare. The EEG results have the potential to be of great importance in practice as simple procedure for the diagnosis of physiological and functional brain disturbances as well as the experimental response to animal reaction for the environmental factors.

The Alternative Housing System for Pigs Growing in Organic Farms

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Abstract: In contrast to conventional systems, organic standards require that animals are kept with outdoor access. Organic pigs farming are poorly developed in Lithuania.

The purpose of this study was to investigate cheap alternative housing system for pigs growing in organic farms in Lithuania.

In 2011 – 2012 years from April 1 to November 1 organic pigs were kept on pasture in camp. From experiment period was investigated the refill farrowing system in the summer camp. Sows were mated in January and were farrowed in summer camp in May – June. Sows with piglets were kept on pasture in camp to November 1 then pigs were moved in pig-house. At the end of the summer period the piglet's weight was 50. 0 ± 10. 0 kg. When in pig-house the pig's weight was 95. 0 ± 5. 0 kg were picked out 30 gilts for mating. Fatting pigs were slaughtered.

In investigation was established in this pig keeping system fit regional pig breeds Lithuanian Native and Lithuanian White. During investigation were analyzed technological parameters: physical environmental factors-during summertime and in pig-house physical, chemical and biological parameters. Were established that pigs keeping technological parameters suit of organic animal husbandry requirements. More space and environmental diversity permitted better expression natural animal behavior with a positive influence on health. Mortality piglets were 2%. It was found that the feed nutritive value during the whole investigation period was less than required for pregnant and lactating sows, piglets. Studies have shown that organically grown Lithuanian White pig breed carcasses qualitative uniqueness, higher pH of the meat, but the pigs growing method less influenced by the composition and meat quality parameters.

Key words: organic pigs, keeping system, camp, meat quality

Effect of Corticosterone on Growth and Welfare of Broiler Chickens Showing Long or Short Tonic Immobility

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Abstract: Tonic immobility (TI) test is commonly used to assess the fear. Animals showing different TI duration demonstrate distinct behavior and biochemical responses to stress. However, less is known how TI phenotype affects growth and welfare of domestic fowls. In this study, broiler chickens were classified into short and long TI duration (STI and LTI) phenotypes and treated chronically with vehicle (CON) or corticosterone (CORT). STI broilers demonstrated significantly higher growth rate with higher breast muscle yield ($P < 0.05$) and lower liver weight ($P = 0.053$) relative to body weight, which was accompanied by higher serum concentration of CORT ($P < 0.05$) and uric acid ($P < 0.01$), but lower serum level of T4 ($P = 0.01$). CORT severely reduced body weight, as well as the relative weight of muscle, bursa of Fabricius and spleen ($P < 0.001$), but the relative liver weight was increased ($P < 0.001$). CORT-treated chickens had reduced serum CORT yet elevated T3, free T3 and heterophile/lymphocyte ratio. STI broilers displayed more preening behavior ($P < 0.05$), and CORT elicited more walking behavior ($P < 0.05$). No difference was observed in the welfare assessment scores between STI and LTI phenotypes under basal situation, while LTI chickens showed significantly increased incidence of pad dermatitis and plumage cleanliness score compared to STI under CORT exposure. The results suggest that STI broilers demonstrate better growth performance and higher adaptability to stress, whereas CORT severely inhibits growth and deteriorates the health and welfare of broiler chickens irrespective of TI phenotype.

Seroprevalence, Hematological and Biochemical Parameters of Brucellosis in Horses

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Abstract: The present study was conducted to determine the seroprevalence of brucellosis in horses in Faisalabad and their hemato-biochemical manifestations. A full detailed anamnestic and clinical assessment in the form of questionnaire was designed for each individual subject. All serum samples were screened for Brucella antibodies by Rose Bengal plate test (RBPT) and serum agglutination test (SAT). Blood samples were evaluated for total erythrocyte counts, hemoglobin concentration, packed cell volume, erythrocytes sedimentation rate, total leukocyte counts, differential leukocytic counts, plasma proteins and fibrinogen following the recommended methods. Sera were subjected to determine alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Results declared that the prevalence of brucellosis was 20.06% in horses by RBPT and 17.15% by SAT. The seroprevalence was significantly ($P < 0.001$) higher in non-lactating mares as compared to lactating mares. Brucellosis does not lead to any significant change in hematological and biochemical parameters in relation to age, sex, body condition and lactation. However, increase in lymphocytes and ALT were observed in seropositive horses.

Studies on Security, Pharmacology and Efficacy of Chinese Herbal Medicine Known as *Qianshiyang*

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Abstract: *Qianshiyang* is made up by *radix asteagali*, *rhizome coptidis*, *tea polyphenols*, *agaricus blazei polysaccharide*, etc. More than 20 kinds of components. It is combined and improved which is according to the animal physiological characteristics and based on anti-cancer chinese herbal medicine compound in people. The research directions are taken by quality control, safety, pharmacological effect, bacteriostasis and antiviral actions. *Radix asteagali* and *rhizome coptidis* are carried out qualitative determination in thin layer chromatography. Baicalin and epicatechin are carried out quantitative determination in liquid chromatography. Acute toxicity test, chronic toxicity test, liver protection, kidney protection, antioxidant effect, immunity researches were made *in vivo* of mouse. The inhibition of Salmonella, resistance of PRV, PRRS was made *in virto*.

(1) In the test of quality control of *Qianshiyang*, astragalus and coptis were carried out qualitative determination in thin layer chromatography, baicalin and epicatechin were carried out quantitative determination in Liquid chromatography. Test results has established a set of detection methods of qualitative and quantitative research on *Qianshiyang*. Baicalin content is 2.0425%, epicatechin content is 0.7152%.

(2) Through the acute toxicity test and chronic toxicity test, *Qianshiyang* has a characteristic of low toxicity and safety range, it is the actually non-toxic material. *Qianshiyang* have a remarkable effect on increasing mice's weight. *Qianshiyang* is a new type of Chinese herbal medicine compound, has a high development potential.

(3) In the liver protection test, three dose groups(0.15 $g \cdot kg^{-1}$, 0.3 $g \cdot kg^{-1}$, 0.6 $g \cdot kg^{-1}$) are setted. The results show that the groups of 0.3 $g \cdot kg^{-1}$ and 0.6 $g \cdot kg^{-1}$ have significant liver protection. In the kindey protection test and immunity test, three dose groups (0.3 $g \cdot kg^{-1}$, 1.5 $g \cdot kg^{-1}$, 3 $g \cdot kg^{-1}$) are setted. The results shows that group of 0.3 $g \cdot kg^{-1}$ has significant effects on protecting kindey and increasing immunity. In the antioxidation test, three dose groups(0.3 $g \cdot kg^{-1}$, 0.6 $g \cdot kg^{-1}$, 1.2 $g \cdot kg^{-1}$) are setted.. The results shows that the three dose groups all have significant antioxidant effect. This research indicate that using ideal dose of *Qianshiyang* is between 0.3 $g \cdot kg^{-1}$ and 1.2 $g \cdot kg^{-1}$.

(4) The test of salmonella inhibition is made by maikangkai medium and nutrient broth. The results shows that concentration of *Qianshiyang* extract is 0.1 $g \cdot mL^{-1}$, it will has the most remarkable bacteriostasis, reach to moderately sensitive level. And bacteriostasis have a certain relevance to the extrat's concentration. The minimum inhibition concentration (MIC) of extract is 0.003125 $g \cdot mL^{-1}$. The test of *Qianshiyang* resist PRV and PRRS is made by PK – 15 and Marc – 15 cells. The results shows that virus are killed in 37℃ keeping 1 h. That is the dose of 0.004 $g \cdot mL^{-1}$ *Qianshiyang* extracts has the role of resistance PRV virus, the dose of 0.002 $g \cdot mL^{-1}$ *Qianshiyang* extract has the role of resistance PRS virus. When less than the concentration, the extract have no antiviral action or lightly antiviral effect.

Key words: *Qianshiyang*, quality standard, security, pharmacological effects

Behavioural, Physiological, Balance and Body Posture Responses of Sheep to Sea Transport Motions

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Abstract: Sheep were exposed to the major types of movement in ships, roll (sideways), heave (vertical) and pitch (fore-aft) to investigate effects on their behaviour, physiology, balance and body posture. Sheep were restrained in pairs in a crate placed on a moveable, programmable platform that replicated the frequency and magnitude of typical ship movements. In Experiment 1 sheep were exposed for 30 minute periods, without food. Heave substantially reduced rumination, but roll caused most stepping motions to correct balance. During heave sheep spent much time with their head above and under the neck/head of the other sheep, with their back against the crate and less time lying down. In experiment 2 sheep were fed to test whether motion sickness caused affected appetite and a mesh was inserted between the sheep to prevent access to the other sheep's feed. They were exposed to the three movements again, with and without an anti-emetic. During heave eating rate was increased and sheep spent more time with their head against the dividing mesh, suggesting that they were steadying themselves. Sheep spent less time with their head on the mesh and against the side bars when they received anti-emetic, suggesting improved balance. We conclude that heave and roll motions have effects on sheep nutritional behaviour and balance which require further investigation.

Hygiene in Animal Production, Cleaning, Disinfection and Deratisation

Effect of Atmospheric Ammonia on the Health and Performance of Broiler Chickens in Summer

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Summary: This study was carried out during summer season on two broiler buildings with a capacity of 10000 each one. The first building with natural ventilation while the second was recently renovated with dynamic ventilation system composed of 10 ventilators with a capacity of 5000 m^3/hour/unit. The results of this experiment showed that ammonia concentration was higher in the building with natural ventilation during various breeding phases. The average concentration of ammonia in the building with natural ventilation was already higher than standard levels from the third week of breeding(16. 5 ppm) and reached higher and alarming levels at the seventh week(31. 5 ppm). Dynamic ventilation in the second building made possible decrease in this concentration that reach 19. 5 ppm at the fourth week and 13. 50 ppm at the end of breeding(week 7). The variation of the ammonia levels in the building with dynamic ventilation is in close relationship to the ventilation flow and working time of the extractor fans. Mortality in the building with natural ventilation was twice higher than that with dynamic ventilation (10. 5% vs. 4. 5%). The presence of dynamic ventilation has also positively influenced the performance parameters of chicken. The day 56, live weight of broiler chickens in the poultry house of dynamic ventilation was superior to that of broiler chickens in building of natural ventilation(1870 vs. 1682 gr). This is confirmed by better feed conversion(1. 98 vs. 2. 66). In the building with natural ventilation, examination of 50 chickens shows the presence of conjunctivitis in animals(20%).

Introduction

The poultry house atmospheric pollution (dusts, ammonia and others gas) are a factor that can influence on production performances and the health of the chicken. Ammonia produced of decomposition and fermentation of the litter and excrements in broiler houses can influence on the growth of chickens and can especially encourage respiratory diseases (Tahseen, 2010). Beker et al. (2004) found that NH_3 in poultry houses lowers performance and may increase disease susceptibility. It has been suggested that NH_3 should not exceed 25 ppm in poultry houses (Carlile, 1984). In Algeria, almost the total parts of buildings are not endowing mechanical ventilation system permitting the optimisation of the temperature and extraction of the toxic gas which is ammonia(NH_3). The goal of our work is to value the effect of ventilation on the concentration of ammonia in two broiler houses in summer. The first is endowed of a dynamic ventilation system whereas the second only possesses natural ventilation. Performances of production and the death rate in every building will also be recorded.

Material and methods

The experimental work was conducted in two broiler houses in the Center of poultry Tazoult(District of Batna) in the summer. The capacities of each poultry house were 10000 chickens; the density was 10 birds/m^2 at the end of broiler breeding.

The first poultry house (P1) had only natural ventilation, composed of four windows(60 cm × 80 cm) on each lateral side and a large gate(180 cm × 210 cm). The second building(P2) was upgraded by installing 10 extractors on a side wall.

The capacity of each fan was 5000 m^3 of air/hour with a flow rate of 4 m^3 air/h/kg live weight in summer. Broiler chickens were raised on a bedof straw(3 kg/m^2). Feed diets were compound of a starter(3050 kcal/kg), growth (3050 kcal/kg) and finishing feed (2900 kcal/kg).

To evaluate the temperature(T) inside the building, we used a manual weather station(Oregon Scientific Bar 938 HG-Model). To measure the concentration of ammonia(NH_3), we used the Gas Sensor(TG-501TOX multi-gas monitor sensor Direct Sense, Gray wolf Sensing solution, 12 Cambridge Drive, Trumbull, CT, 06611 USA).

The air velocity(V) in the buildings was removed by Manometer type digital Testo 415 GmbH Germany.

These parameters were recorded every day until the end of breeding, according to the method of Rokicki and Kolbuszewski, 1996. The ventilation flow in broiler house (P2) was calculated to determine the working time of extractors and ventilation rate for each rearing phase. In parallel, we recorded the performance parameters(feed intake, weight gain, feed conversion and mortality). The

statistical analysis was performed using ANOVA and MS Excel file 2009.

Results and discussion

The microclimatic conditions in the two broilers houses (P1, P2), are indicated in Table 1. The variations of the internal temperature were due to the ventilation system applied in the buildings during various phase of breeding.

Mechanical ventilation influenced the ammonia rate positively in the P2 building.

During the first three weeks, the ammonia rate in the two buildings was in conformity with the standards norms (Le Menec, 1984; Rokicki and Kolbusewski, 1996, Ritz et al., 2004). The maximum value ammonia was 16.65 and 10.25 ppm respectively in the two buildings. By the end of the fourth week of breeding, this rate increased in the P1 building. It varied between 22.15 and 31.20 ppm, until the end of breeding.

In the P2 building, mechanical ventilation involved a reduction in this toxic gas, from 19.45 to 13.50 ppm at the end of the breeding. These results agree with several works (Reece et al., 1980; Miles et al., 2002; Redwine et al., 2002).

Contrary to the building P1, the mechanical system ventilation in the P2 building, influenced the internal temperature positively. Between the fourth and the seventh week of breeding, the internal temperatures in the P2 building were largely lower than these of the P1 building in which an inappropriate ventilation in this last poultry house, was characterized by a low air velocity.

The flow of ventilation in the P2 building increased according to the breeding phases which induced a consequent increase air velocity. The work of Demmers et al., (1999) confirms the effectiveness of mechanical ventilation in the optimization of the temperature and toxic gases (NH_3 and CO_2).

The evolution of the ammonia rate in the two buildings is represented by Fig. 1. In the P1 building, the curve is ascending according to the duration of breeding (7 week). However in the P2 building, the curve began with an ascending phase up to the value from 19.5 ppm of ammonia then is declined to a point where the rate of ammonia is 12.50 ppm.

The production performances in J49 are summarized in Table 2. The final weight of chickens in the P2 building was higher than those of the P1 building (1870 vs 1682 gr.). The quantity of feed consumed by chicken in the P1 building was higher 780 gr. than that consumed in the P2 building This is reflected in a lower feed conversion in broiler house P2 (2.66 vs 1.98). Current results are in agreement with the previous research work of Charles and Payne (1966), Reece and Lott (1980) and Beker et al. (2004). The death rates in the building with natural ventilation were twice higher than that of the building with mechanical ventilation. Several authors (Christensen et al., 2000; Al Homidani et al., 2003) confirm this hypothesis. In the building with natural ventilation, examination of 50 chickens shows the presence of conjunctivitis in animals (20%).

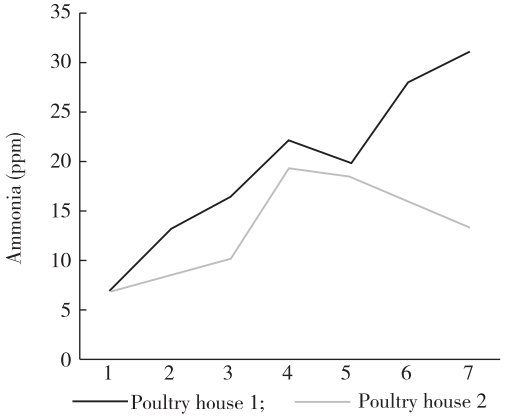

Fig. 1 Ammonia levels in broiler houses for seven weeks

Table 1 Ventilation conditions, temperature and ammonia (NH_3) in broiler houses

Week	Broiler house (P1)				Broiler house (P2)			
	Ammonia (ppm)	Air velocity (m/s)	Ventilation (m^3/h)	Temperature (℃)	Ammonia (ppm)	Air velocity (m/s)	Ventilation (m^3/h)	Temperature (℃)
1	7.10 ± 0.4	0.11 ± 0.06	-	33.30 ± 2.25	6.80 ± 0.08	0.62 ± 0.05	4160	31.50 ± 5.15
2	13.20 ± 1.6	0.41 ± 0.03	-	31.90 ± 3.75	8.50 ± 0.06	0.45 ± 0.07	7400	28.50 ± 2.75
3	16.65 ± 2.2	0.24 ± 0.05	-	30.25 ± 2.70	10.25 ± 0.06	0.32 ± 0.09	15200	28.75 ± 3.50
4	22.15 ± 0.15	0.48 ± 0.07	-	29.65 ± 4.50	19.45 ± 1.02	0.70 ± 0.06	29200	27.50 ± 4.50
5	19.90 ± 2.25	0.22 ± 0.02	-	30.00 ± 2.50	18.50 ± 0.09	0.57 ± 0.08	35200	27.00 ± 2.25
6	28.10 ± 3.25	0.49 ± 0.03	-	28.50 ± 3.45	15.75 ± 2.06	0.85 ± 0.04	48400	25.50 ± 3.75
7	31.20 ± 2.5	0.25 ± 0.04	-	30.00 ± 5.10	13.50 ± 1.26	0.75 ± 0.09	74800	23.50 ± 2.50

Table 2 Performance of production and mortality in broiler houses

Broiler house	Capacity(birds)	Bird/m^2	Feed intake(gr)	Live weight(gr)	Feed conversion	Mortality(%)
P1	10000	10	4480 ± 132	1682 ± 79	2.66	10.5
P2	10000	10	3700 ± 104	1870 ± 56	1.98	4.8

Conclusions

Mechanical ventilation is a process which it is necessary to install in all the broiler houses because it involves an improvement of the internal environment parameters of the building in summer: an increase in the air flow and its velocity, a decrease in high temperatures in poultry houses, a reduction of the concentration of ammonia, an improvement of production performances, a reduction of the death rate.

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Feed Technology and Health of Foot Pads in Broilers

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Summary: This study aimed to test potential effects of the physical form of diets based on same ingredients and chemical composition on the health of foot pads in broilers. Three consecutive trials were done with 306 of one-week-old broiler being reared over 4 weeks. In each trial, the birds were divided randomly into 4 groups. Each floor pen was littered with wood shavings (1 kg/m^2). All birds were fed *ad libitum* a diet based on identical ingredients (%, 64 wheat; 30 toasted soybean; 2 soy oil; 2.5 mineral and vitamin mixtures and 1.5 $CaCO_3$). For diet 1 ingredients were ground finely (hammer mill, h. m.), for diet 2 ground coarsely (roller mill, r. m.), diet 3 contained 22% unground wheat, diet 4 was prepared as an extrudate. However, all diets had about an identical chemical composition. External assessment of foot pads was done weekly.

Inspite of identical ingredients and identical chemical composition of the diet there were significant effects of feed technology. Extrudate diet resulted in increased development and severity of foot pad dermatitis (FPD) whereas the finely ground pelleted diet decreased it significantly. No significant differences of FPD scores were found between diets 2 and 3. It has to be emphasized that severity of FPD scores was correlated to dry matter contents of the litter (estimated at the end of 3 trials in whole litter). It is supposed that the effects of feed technology are mediated by changes in the water: feed intake ratio and its consequences on the dry matter content of the litter; presumably there are further effects due to changes in the water release from the litter (drying of the litter surface?); this property of the litter could also be influenced by the water binding capacity of undigested carbohydrates.

Introduction

Foot pad dermatitis (FPD) is a widespread problem in poultry production and is a potential welfare and economic problem in intensive production systems. The low prevalence and severity of FPD are of great concern regarding animal welfare, the birds' performance and product quality. Thus recently, the condition of poultry feet is used as a production criterion to evaluate the animal welfare programs implemented by commercial poultry companies. Factors affecting the incidence and severity of FPD have been reviewed by KAMPHUES et al. [2]. However, quality of litter, especially its moisture content, is of special interest regarding feeding measures and housing conditions to reduce incidence of FPD. The control of excreta/litter moisture and quality is a priority in the modern poultry production, to avoid animal welfare problems.

A variety of technological processes for treatment of feed ingredients and compounded feeds are available for the feed industry. Feed processing technology plays a significant role since it enables improve in the nutrient digestibility, reduction of antinutritive factors and decrease of the feeds' technology (hygienic treatment) as it was reviewed by PEISKER [4].

However, the feed technology (type of grinding, compaction, thermal treatment) differs among feed producers depending on preferred ingredients or "treatment" of feedstuffs and that technology could have an effect on the incidence of FPD through excreta and/or litter quality. However, little is known about effect of feed technology on the health of foot pads. Thus, this study aimed to test potential effects of the physical form of diets based on identical ingredients and chemical composition on the health of foot pads in broilers.

Material and methods

Three consecutive trials were done with 306 birds of one-week-old ♂ broiler (Ross 708) being reared over 4 weeks (d 8 – 36). At the beginning of the experimental period (d 8), in each trial the birds were divided randomly into 4 groups (25 or 26 bird/group). Each floor pen (1.40 m × 0.85 m) was littered with wood shavings (1 kg/m^2; 87.3% ± 0.2% dry matter). All birds were fed *ad libitum* a diet based on identical ingredients (%, 64 wheat; 30 soybean meal; 2 soy oil; 2.5 mineral and vitamin mixtures and 1.5 $CaCO_3$). For diet 1 ingredients were ground finely (hammer mill, h. m.), for diet 2 ground coarsely (roller mill, r. m.), diet 3 contained 22% unground wheat "was also pelleted", diet 4 was prepared as extrudate. However, all diets had about an identical chemical composition. It has to be emphasised

that 2 kg fresh clean dry litter was added for all the groups during the whole experimental period (to avoid any negative effect of litter on the results). Litter samples for measuring DM and pH were collected at d 8, 15, 22, 29, and 36. Body weight was recorded weekly at the same day of scoring individually. Feed and water intakes were measured daily at group level. Feed conversion ratio (FCR) was estimated on the basis of feed consumed (data from groups) and weight gain of the birds (individual data) throughout the experimental period. The external examination was done for birds at the beginning of the experiment (d 8), then weekly till d 36. If the feet were dirty, they were gently washed with a wet cloth and dried before scoring; only the central plantar was scored, signs of foot pad lesions were recorded on a 7-point scale (0 = normal skin; 7 = over half of the foot pad is covered with necrotic scales) according to MAYNE et al. [3].

Results

Birds fed extrudate diet had the highest numerical water: feed intake ratio (2.67) compared to the other experimental groups. However, it was observed that the lowest numerical water: feed intake ration was for birds pellet fine/unground diets (2.57). Table 1 shows that end body weights of birds that were fed pellet fine or pellet fine/unground were increase significantly (2374 and 2371 g, respectively) compared to the other experimental groups (2109 g for pellet coarse and 2198 g for extrudate). Moreover, favourable FCR were achieved by feeding pellet fine or pellet fine/unground diets (1.49 and 1.50, respectively) in comparison to the other experimental diets (1.59 for pellet coarse and 1.60 for extrudate).

Table 1 Comparison of broiler's body weight (g) at different times

Diet	Day (duration of treatments)					FCR
	8 (0) (n = 77)	15 (7) (n = 76)	22 (14) (n = 74)	29 (21) (n = 52)	36 (28) (n = 42)	
Pellet fine	$157^{A} \pm 25.3^{1)}$	$455^{A} \pm 79.5^{2)}$	$917^{A} \pm 155^{3)}$	$1617^{A} \pm 226^{4)}$	$2374^{A} \pm 308^{6)}$	1.49
Pellet coarse	$155^{A} \pm 28.8^{1)}$	$417^{B} \pm 64.8$	$827^{B} \pm 128$	$1433^{B} \pm 188$	$2109^{B} \pm 234$	1.59
Pellet fine/unground	$158^{A} \pm 29.2$	$452^{A} \pm 82.2$	$911^{A} \pm 151$	$1608^{A} \pm 196$	$2371^{A} \pm 266^{7)}$	1.50
Extrudate	$160^{A} \pm 28.6$	$426^{B} \pm 67.3$	$853^{B} \pm 117$	$1463^{B} \pm 248^{5)}$	$2198^{B} \pm 250$	1.60

^{A,B}Means in the same column with different superscripts are significantly different ($P < 0.05$).
$^{1)}$n = 76; $^{2)}$n = 75; $^{3)}$n = 73; $^{4)}$n = 51; $^{5)}$n = 53; $^{6)}$n = 38; $^{7)}$n = 41

At the beginning of the experiment (d 8) there was no evidence of external FPD lesions. Table 2 shows that at the end of the experimental period (d 36), birds fed pelleted fine diet had significantly lower external FPD scores (2.98 ± 1.11) compared to other groups. Furthermore, no significant differences of external FPD at d 36 were observed between birds fed pellet coarse or pellet fine/unground (3.77 ± 0.706 and 3.71 ± 0.928, respectively). Interestingly, external FPD scores of birds that were fed extrudate diet were significantly higher (4.53 ± 1.22) in comparison to other groups.

Table 2 Development of external foot pad scores of broilers during the experimental period (Mean ± SD)

Diet	Day (duration of treatments)/FPD scores			
	15 (7) (n = 76)	22 (14) (n = 74)	29 (21) (n = 52)	36 (28) (n = 42)
Pellet fine	$0.593^{B} \pm 0.498^{1)}$	$2.01^{C} \pm 1.07^{2)}$	$2.33^{C} \pm 1.24^{3)}$	$2.98^{C} \pm 1.11^{5)}$
Pellet coarse	$0.737^{B} \pm 0.404$	$2.54^{AB} \pm 1.13$	$2.99^{B} \pm 0.905$	$3.77^{B} \pm 0.706$
Pellet fine/unground	$0.769^{B} \pm 0.369$	$2.36^{BC} \pm 1.09$	$3.02^{B} \pm 0.939$	$3.71^{B} \pm 0.928^{6)}$
Extrudate	$1.11^{A} \pm 0.585$	$2.87^{A} \pm 1.03$	$3.59^{A} \pm 0.915^{4)}$	$4.53^{A} \pm 1.22$

^{A,B}Means in the same column with different superscripts are significantly different ($P < 0.05$).
$^{1)}$n = 75; $^{2)}$n = 73; $^{3)}$n = 51; $^{4)}$n = 53; $^{5)}$n = 38; $^{6)}$n = 41

Discussion

Today it is usual practice to process poultry feeds by means of pelleting or even expansion and extrusion aiming to reduce the risk of pathogenic microoganisms contamination. However, caution should be exercised when choosing manufacture techniques so that pellet quality improvement does not compromise litter quality and hence health of foot pads.

The current industry practice of using highly processed, pelleted diets masks the influence of particle size, but some reports suggest that the effects of feed particle size on performance may be maintained even after pelleting [1]. The present data shows an obvious effect of pelleting on the end body weight of the broilers. Feeding birds pellet fine and or pellet fine/unground diets

were associated with significantly higher body weight. In contrast, a number of studies have shown that feed particle size has no significant effect on broiler performance in pelleted feeds. In previous studies, REECE et al. [5] found no effect on performance using maize of differing particle sizes to formulate broiler starter diets in crumble form. Similarly, SVIHUS et al. [7] showed no difference in any of the performance parameters when broilers were fed pelleted feeds made from wheat ground in hammer and roller mills to a range of particle sizes and concluded that pelleting evened out differences in particle size distribution. Feeding different physical forms of diets seems to have a significant effect on DM content of the litter as was observed in this study. However, no significant differences were found in the DM contents of excreta in all experimental groups. The relationship between water and feed intake ratio was only slightly higher for birds fed extrudate diets. Therefore, the forced water intake caused by that diet could be neglected. However, the release of water from litter and/or excreta could an interesting point. The present data shows significant effects of feeding different physical forms (although of the identical chemical composition among the treatments) were observed on the FPD scores. FPD scores of birds that were fed extrudate diet were significantly higher compared to other experimental groups. These results may suggest that temperature and pressure in the extrudate diet could influence on the digestibility of nutrients and hence on the excreta quality.

It is supposed that diets' physical form effects are mediated by changes in the water:feed intake ratio and its consequences on the dry matter content of the litter; presumably there are further effects due to changes in the water release from the litter (drying of the litter surface?); this raises the question of whether this property of the litter could also be influenced by the water binding capacity of undigested carbohydrates (non starch polysaccharides? Here, no dietary enzymes were added).

Conclusions

This study highlights the effect of feed technology on the litter quality and hence on the incidence of FPD in broilers. From another point of view, feed manufacturing is an expensive process, both in terms of capital investment and running cost, in particular expenditures for energy. It could be assumed that pelleting increases cost of the feed by at least 4% compared with mash [6]. Therefore, the aims connected with feed processing should me met at the highest possible level. These aims can be condensed to flexibility of ingredient usage, improving dietary feeding/digestibility and hence excreta and litter quality. More research is necessary to elucidate the effects of high temperature and pressure on the changes of NSP "water binding capacity" and hence on the excreta and/or litter quality.

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Do Floor Heating and Litter Moisture Affect the Outcome of an Artificial Coccidial Infection in Young Turkeys Fed a Diet without Anticoccidia?

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Summary: An objective of the current study was to determine the effects of floor heating when poults were artificially infected with Eimeria regarding the outcome of the infection and secondary effects on litter quality and health of the foot pad. Two trials were done, in each four groups of 2-week-old turkeys being reared during 4 weeks. All birds were fed one identical pelleted diet without any anticoccidial additive. The first 2 groups were kept on *dry* wood shavings, with and without floor heating, the 3rd/4th group on *wet* wood shavings (~35% water), with and without floor heating. In each group two birds "seeder" only were infected experimentally (~50,000 oocysts of *E. adenoeides*/bird) and nominated as primary infected, the other were nominated as secondary infected birds. At trials' end each bird was scored macroscopically for coccidial lesions in the cecum as well as for oocysts counts (Log_{10}) in cecal contents. Finally, the foot pads were assessed weekly.

Using floor heating *without* exposure to *wet* litter in both trials reduced counts of oocysts in excreta of primary infected birds (1.52/1.21; 0/1.82 and 1.69/0; trials 1/2, at 16, 20, and 24 d post inoculation). Nevertheless, in both trials *using floor heating with* exposure to wet litter resulted in higher counts of oocysts in the excreta of secondary infected birds (3.72/3.92; trials 1/2) at d 24 post inoculation compared with the other groups. Using floor heating resulted in significantly decreased foot pad dermatitis scores compared with groups housed without floor heating. It may concluded that when diet composition affects the litter quality, there are secondary effects on the process of Eimeria infection tested in a model with experimentally applied oocysts.

Introduction

Coccidiosis is one of the most important and common diseases that affect poultry, it results in great economic loss all over the world [4]. In turkeys seven species of *Eimeria* have been reported. However, *Eimeria adenoeides* (*E. adenoeides*) is considered the most pathogenic, infecting the cecum of birds [8]. It is generally believed that bad management such as wet litter will favour the development of coccidiosis, because of the higher sporulation ability thus induced [5]. Sporulation of the oocysts depends mainly on three basic factors: temperature, humidity and access to oxygen [9], thus quality of litter especially its moisture content, is of special interest regarding feeding measures and housing conditions to reduce incidence of foot pad dermatitis (FPD). FPD can achieve a prevalence of approximately 20% for severe lesions and 78% for mild lesions in fattening turkeys [3]. At the end of the turkeys' fattening period, this disease could reach a prevalence of 91% – 100% [7]. The prevalence of FPD per farm is gaining recognition as a well-being indicator [3]. In light of the above-mentioned literature, the current study was focused on the outcome of an artificial infection in young turkeys with *E. adenoeides* under the influence of floor heating combined (+) with or without "critical moisture content" (water content of ~35%) of the litter, with special regard on foot pad health.

Material and methods

Two experimental trials were performed. In each trial, ten birds were chosen randomly for necropsy at the beginning of the experimental period (d 14) for foot pad histopathological assessment. The remaining birds, 80 in total in each trial, were individually identified and then divided into 4 equal groups, each housed in a floor pen (1.50 m × 1.32 m). The first 2 groups were kept on "dry" wood shavings with and without floor heating; the other 2 groups were housed on wood shavings with a moisture content of 35%, with and without floor heating. The electrical floor heating system supplied with an adjuster to control the temperature was used. Coccidial infections were established by means of seeder birds. Thus, only two birds in each group (nominated as primary infected birds) were experimentally infected with a pure isolate containing *E. adenoeides* via crop intubation with 1 ml of ~50,000 sporulated oocysts/bird. It has to be emphasized that each bird in all groups was marked

individually during the experimental period. The number of oocysts produced in the excreta of primary infected birds was determined after each 4 d post inoculation (PI) until the end of the experiment. In each group, the two primary infected birds were taken out of the pen during the time of collection pooled excreta of the other ones (nominated as secondary infected birds) at 8, 12, 16, 20 and 24 days PI, then those two primary infected birds returned back to the pen. Also at the same day of collecting the pooled excreta, the samples from each 6 secondary infected birds/group were collected for counting the oocysts. It should be stressed here that the 6 secondary infected birds were randomly chosen every time. A clean polyethylene sheet covering the litter was used for collecting pooled excreta ~ 100 g/group for oocyst counting according to Hodgson (1970). External assessment of foot pads was done at d 14, 21, 28, 35 and 42. At d 42 all birds were sacrificed for microscopic evaluation of foot pads. During the external examination, if the feet were dirty, they were gently washed with a wet cloth and dried before scoring; only the central plantar was scored, signs of foot pad lesions were recorded according to MAYNE et al. [11].

Results

Table 1 shows the effects of floor heating as well as daily exposure to wet litter on the oocyst counts in the excreta of primary infected birds in both trials. Using floor heating without exposure to wet litter in both trials reduced the oocyst numbers in the excreta (1.52/1.21; 0/1.82 and 1.69/0 in trials 1/2 respectively at 16, 20, and 24 days PI).

Table 2 shows the effects of floor heating as well as daily exposure to wet litter on the oocyst counts in the excreta of secondary individual infected birds in both trials. In both trials, using floor heating without exposure to wet litter was associated with a marked decrease in oocyst counts (d 12 till d 24 PI) compared with the other experimental groups.

Table 1 Oocyst counting (Log 10/g excreta) of primary individual infected birds for both trials. According to ABD EL-WAHAB et al. [2]

Floor heating	Exposure to wet litter (h/d)	Day (post inoculation)/primary						
		4 (n = 2/2)	6 (n = 2/2)	8 (n = 2/2)	12 (n = 2/2)	16 (n = 2/2)	20 (n = 2/2)	24 (n = 2/2)
-	0	2.63/0	4.75/4.12	2.26/4.05	4.47/3.05	1.82/2.69	2.39/2.65	2.36/2.56
-	24	2.60/1.22	4.79/5.02	2.36/1.52[1)]	3.49/4.66[1)]	1.99/4.10[1)]	3.42/3.77[1)]	3.08/2.73[1)]
+	0	2.58/0	4.86/4.90	3.23/3.48	3.69/0	1.52/1.21	0/1.82	1.69/0
+	24	2.50/1.22	4.19/5.94	2.79/4.56[1)]	3.45/4.44[1)]	1.52/1.51[1)]	3.34/3.11[1)]	3.18/3.76[1)]

No statistical analysis due to small number of samples.
[1)] n = 1

Table 2 Oocyst counting (Log 10/g excreta) of secondary individual infected birds for both trials. According to ABD EL-WAHAB et al. [2]

Floor heating	Exposure to wet litter (h/d)	Day (post inoculation)/secondary				
		8 (n = 6/6)	12 (n = 6/6)	16 (n = 6/6)	20 (n = 6/6)	24 (n = 6/6)
-	0	$1.45^{a}/0.000^{b}$	$1.37^{ab}/2.19^{a}$	$3.69^{a}/3.98^{a}$	$1.76^{a}/2.40^{a}$	$2.30^{a}/1.64^{ab}$
-	24	$1.30^{a}/2.19^{a}$	$2.45^{a}/3.68^{a}$	$2.53^{ab}/3.66^{a}$	$0.737^{ab}/1.99^{a}$	$2.36^{a}/2.63^{a}$
+	0	$0.811^{a}/0.395^{b}$	$0.811^{b}/0.000^{b}$	$0.507^{c}/0.556^{c}$	$0.254^{b}/0.606^{b}$	$0.304^{b}/0.354^{b}$
+	24	$1.47^{a}/0.304^{b}$	$2.11^{ab}/2.61^{a}$	$1.04^{bc}/2.33^{b}$	$0.607^{ab}/1.42^{ab}$	$2.54^{a}/2.49^{a}$

[a,b] Means in the same column in each trial with same superscripts are not significantly different ($P < 0.05$).

Furthermore, in order to provide more details on the effects of the severity of coccidial infection on mean DM content of excreta, the oocyst counting "Log10/g excreta" was classified into 3 categories (numbers 0 – 2 = low; numbers 2 – 3.5 = medium and numbers 3.5 – 5 = high). Accordingly, it was observed that in both trials the low counts of coccidia were accompanied by significantly increased DM content of excreta (17.4% ± 1.11% and 17.5% ± 0.568% in trials 1 and 2, respectively) vs. (14.5% ± 0.900% and 14.6% ± 1.10% in trials 1 and 2, respectively) for the high counts of oocyst in excreta.

Table 3 shows that both trials using floor heating resulted in significantly decreased external FPD scores (2.06 ± 0.735 and 1.47 ± 0.734, trials 1 and 2 respectively) in comparison to groups without floor heating (3.88 ± 0.812 and 2.73 ± 1.25 in trials 1 and 2 respectively for external scores).

Table 3 External and histopathological foot pad scores (two factor variance analyses; mean ± SD). According to ABD EL-WAHAB et al. [2]

	Factor	Treatment	Day (duration of treatments)/FPD scores				
			External				Histopathology
			21(7) (n=40)	28(14) (n=40)	35(21) (n=40)	42(28) (n=40)	42(28) (n=40)
Trial 1	Floor heating	−	$1.38^{az} \pm 0.711$	$2.08^{ay} \pm 0.897$	$3.02^{ax} \pm 1.27$	$3.88^{aw} \pm 0.812$	$3.53^{a} \pm 1.07$
		+	$0.825^{bz} \pm 0.572$	$1.36^{by} \pm 0.518$	$1.60^{bx} \pm 0.622$	$2.06^{bw} \pm 0.735$	$2.06^{b} \pm 0.662$
	Exposure to wet litter (h)	0	$0.837^{bz} \pm 0.485$	$1.45^{by} \pm 0.522$	$1.80^{bx} \pm 0.822$	$2.53^{bw} \pm 1.00$	$2.41^{b} \pm 0.979$
		24	$1.37^{az} \pm 0.782$	$2.00^{ay} \pm 0.940$	$2.82^{ax} \pm 1.35$	$3.41^{aw} \pm 1.23$	$3.18^{a} \pm 1.20$
Trial 2	Floor heating	−[1]	$0.935^{az} \pm 0.400$	$1.21^{ay} \pm 0.540$	$2.06^{ax} \pm 1.11$	$2.73^{aw} \pm 1.25$	$2.24^{a} \pm 0.841$
		+[1]	$0.410^{bz} \pm 0.427$	$0.782^{by} \pm 0.410$	$1.02^{bx} \pm 0.458$	$1.47^{bw} \pm 0.734$	$1.51^{b} \pm 0.493$
	Exposure to wet litter (h)	0	$0.512^{bz} \pm 0.486$	$0.950^{ay} \pm 0.421$	$1.15^{bx} \pm 0.521$	$1.53^{bw} \pm 0.683$	$1.56^{b} \pm 0.579$
		24[1]	$0.842^{az} \pm 0.436$	$1.04^{ay} \pm 0.616$	$1.96^{ax} \pm 1.19$	$2.69^{aw} \pm 1.34$	$2.21^{a} \pm 0.827$

[a,b] Means in the same column within each criteria in each trial with different superscripts are significantly different ($P < 0.05$).
[w,x,y,z] Means in the same row within each criteria in each trial with different superscripts are significantly different ($P < 0.05$). [1] n = 39

Discussion

It is well documented that moist conditions in litter favour outbreaks of coccidiosis, and one of the factors is believed to faster sporulation [5]. DAVIES and JOYNER [6] observed a positive correlation between oocysts and litter moisture. This explained clearly the highest counts of oocyst in the excreta were found only in the birds exposed to wet litter. Additionally, combination of floor heating and dry litter resulted in markedly reduced oocyst counts in primary and secondary infected birds. However, WALDENSTEDT et al. [12] observed that sporulation rate was higher in a dry environment (16% moisture content) than in moist (62% moisture content) indicating that the increased frequency of outbreaks of coccidiosis in broiler reared under moist conditions might be due to factors other than improved oocyst sporulation. One likely factor is the survival time of sporulated oocysts. MAROQUARDT et al. [10] observed that at 35℃ sporulation was morphologically abnormal and the oocysts would not be infectious. Nevertheless, using floor heating with experimentally wet litter resulted in high counts of oocyst. It means that both factors (temperature and moisture) act additively on the development of coccidiosis.

A low prevalence and severity of foot pad dermatitis (FPD) are highly desirable regarding the health of birds and product quality. FPD can be kept at a minimum with proper litter management. Thus, with increasing prevalence and severity of FPD on farms, intestinal infections, such as coccidiosis should not be neglected. Despite diarrhoea induced by coccidial infection, the litter became drier when floor heating was used. Therefore, floor heating is likely to be highly effective in reducing the development and severity of FPD. In recent experiment [1] it was noted that the significant effect of using floor heating on FPD scores could be due to the litter becoming dry as fresh litter or could be due to floor heating leading to warm foot pads causing vasodilatation of the blood vessels. It means avoiding vasoconstriction and the low blood flow due to cold foot pads.

Conclusions

The present results suggest that floor heating affected the process of infection (shedding of oocysts) and the health of foot pad.

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Efficacy of Some Disinfectants against Avian Influenza Virus in Layer Farms

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Summary: This study was carried out in 6 layer houses at Giza province, during outbreaks of high pathogenic avian influenza(HPAI) diagnosed in Egypt. The present investigation was undertaken to evaluate the viricidal activity of different disinfectants against avian Influenza virus(AIV) under laboratory and field conditions. Virkon S 1% and Anolyte(1/250) were the most effective disinfectants in killing AIV. Despite the good results obtained with Aldekol 0.5%, Longlife 250 S 0.5% and TH4 0.5% in laboratory test after 10 min, but the effect of both disinfectants on AIV infected premises was failed.

Introduction

Studies for the determination of the efficacy of chemical substances (Haussmann and Grafe, 1957; Henneberg and Hoppner 1960; Sprossig and Mucke, 1968; King, 1991; Davison et al., 1999; Muhmmad et al., 2001; Yilmaz et al., 2005) demonstrated a high sensitivity of influenza viruses, but the test conditions chosen were not very suitable for evaluating the efficacy of disinfectants against IVA in animal husbandry.

Documentation of the effectiveness of viral disinfectants is minimal, and even less information is available on the mechanism of action and their efficacy in the presence of organic challenge. In addition to the lack of efficacy data, the data that are available in the literature are difficult to interpret and compare against other data due to lack of standardized testing protocols for the inactivation of viruses.

The objective of the present study was to evaluate the efficacy of different disinfectants for control of AIV either by laboratory and field tests.

Materials and methods

Materials

Samples:

Swab samples were collected from 6 premises of commercial layers houses at Giza province. The virus confirmed after submission to the reference laboratory (National Laboratory for Veterinary Quality Control of Poultry Production) for characterization(HPAI H5N1).

Disinfectants:

1 – Aldekol des 03, (Germany), the recommended concentration is 1 L/200 L(0.5%).

2 – Longlife 250 S, (UK), it was used at a concentration of 0.5%.

3 – TH4(Sogeval, Laval-France), it was used at a concentration of 0.5%.

4 – Virkon S (Dupont, UK), it was used at a concentration of 1%.

5 – Anolyte(Envirolyte, Germany), pH from 2.0 to 8.5, 1\500 = 2 mg/L active chlorine, 1\1000 = 1 mg/L active chlorine.). The tested protocol was followed according to Suarez et al., (2003).

Antigen detection tests:

Antigen Rapid AIV Ag Test Kit used for detection of AIV in avian droppings, with a high degree of accuracy. (Institute Animal Genetics, Inc. of Korea).

Embryonated specific pathogen free (SPF) chicken eggs:

11-day-old chickens' embroynated eggs were used for propagation of AIV and confirmation of the results of Antigen Rapid AIV AG Test(OIE, 2005).

Methods

Virus propagation:

H5N1 virus was propagated in 11 day old chicken embryonated eggs(CEE) where, tenfold virus dilutions were inoculated into 11-day-old chicken embryonated eggs in six-replications. and then incubated in a 37℃ humidified incubator and candled twice a day for 7 days. The ELD_{50}/ml evaluated according to the method described by Reed and Muench(1938).

Virus identification and characterization:

The infectious AF was harvested from each CEE and subjected to HA and HI test according to OIE standard methods(OIE, 2005).

Laboratory trials

Laboratory evaluation of the disinfectants:

The tested protocol was followed according to Suarez et al., (2003) and Wanaratana et al., (2010). About a 0.5 ml of AIV containing approximately 1.0×10^6 ELD_{50}

was mixed with 0. 5 ml of diluted disinfectants and incubated for 10 min and 1 hr at room temperature. After 10 min and 1 hr incubation period, 0. 1 ml of the virus-disinfectant mixture, the positive and negative controls were inoculated into 11-day-old CEE in three replications. The allantoic fluid was harvested from each egg on day 7 post inoculation.

Field Trials

Evaluation of disinfectants on the poultry house floor:

A poultry farm infected with high pathogenic avian influenza (HPAI) was chosen for carrying out the field trial. Experimental test units were 1-ft2 floor plots. A half ml of AIVs containing approximately 1.0×10^6 ELD_{50} was placed in the center of the plot. Each disinfectant was applied to 10 plots as a coarse spray at a low application rate of 125 ml/plot. Five untreated plots, receiving no disinfectant, served as negative control and also another five virus treated plots, served as positive control.

Evaluation of disinfectants in the poultry house:

Sex AIV infected houses were used in this trial, after removal of the droppings, the houses were dry-cleaned and wet cleaned. Swabs were taken from different parts of the poultry houses to detect the presence of the virus. Samples that give a negative reaction should be passage into at least one further batch of eggs (OIE, 2005).

Result and discussion

Previous investigations on AIV disinfection performed with different substances in suspension tests with and without organic load or on carriers like line or batiste gave important information on the effects of disinfectants against AIV (Haussmann and Grafe, 1956; Albrecht, 1957; Albrecht et al., 1957/58; Henneberg und Hoppner, 1960; Horn, 1960; Sprossig and Mucke, 1968; King, 1991). However, most of the disinfectants tested in these studies are not very common nowadays, and the methods used were not very suitable for testing the ability of a disinfectant for veterinary field conditions. Especially in animal husbandry, the requirements on a disinfectant are very high, as a lot of factors like high organic soiling even after proper cleaning, different materials with often porous surfaces, low temperatures and short contact times can negatively influence its efficacy.

The results from the present study indicated that H5N1 isolated virus could be moderately inactivated by exposure to the disinfectants including, Aldekol des 03 0. 5%, Virkon S 1%, Longlife 250 S 0. 5%, TH4 0. 5% and Anolyte (1/500) for 10 min. The Anolyte at concentration of (1/250) was superior for the complete inactivation of high pathogenic avian influenza virus than the other disinfectants (Table 1).

The results of evaluation of disinfectants on the poultry house floor showed that the disinfectant Anolyte could completely inactivate H5N1 virus when used at concentration of (1/250). Other disinfectants Aldekol des 03, Virkon S, Longlife 250 S and TH4 could efficiently inactivate H5N1 virus when used at the manufacturer's recommended concentration for 1 hr (Table 2).

Table 1 Laboratory evaluation of the disinfectants

Disinfectant	Time of exposure			
	10 min	1 hr	+ ve Control	– ve Control
Aldekol des 03 0. 5%	2#:6	0:6	5:6	0:6
Virkon S 1%	2:6	0:6	5:6	0:6
Longlife 250 S 0. 5%	2:6	1:6	6:6	0:6
TH4 0. 5%	3:6	2:6	5:6	0:6
Anolyte (1/500)+	3:6	2:6	6:6	0:6
Anolyte (1/250)++	0:6	0:6	6:6	0:6

+:2 mg/L active chlorine, ++:4 mg/L active chlorine. #: Virus propagation.

Table 2 Evaluation of disinfectants on floor house

Disinfectan	Time of exposure			
	10 min	1 hr	+ ve Control	– ve Control
Aldekol des 03 0. 5%	2#:6	0:6	5:6	1:6
Virkon S 1%	2:6	0:6	5:6	1:6
Longlife 250 S 0. 5%	2:6	0:6	6:6	2:6
TH4 0. 5%	3:6	0:6	5:6	0:6
Anolyte (1/500)+	3:6	2:6	6:6	1:6
Anolyte (1/250)++	0:6	0:6	5:6	0:6

+:2 mg/L active chlorine, ++:4mg/L active chlorine. #:Virus propagation.

The results of viricidal effect of tested disinfectants on avian influenza virus infected poultry premises in Table 3, showed that Virkon S (1%) and Anolyte (1/250) were very effective in complete killing of H5N1 viruses. These results coincided with the results recorded by Suarez et al (2003) concerning the Virkon S 1%. It was also noticed that Long life (0.5%) in houses failed to control AIV even after the third application at 8th day. Aldekol 0.5%, TH_4 0.5% and Anolyte (1/500) at 4th day after application in houses gave complete sanitation of the houses from AIV.

Table 3 Viricidal effect of tested disinfectants on avian influenza virus infected poultry premises

Disinfectants group	Aldekol 0.5%	Virkon S 1%	Longlife 0.5%	TH4 0.5%	Anolyte (1/500)	Anolyte (1/250)
Houses	1st house	2nd house	3rd house	4th house	5th house	6th house
Times						
10 minutes	V	N	V	V	V	N
4th Day	N	-	V	N	N	-
8th Day	-	-	V	-	-	-

N: No virus detected; V: Virus detected

Conclusion

Virkon S 1% and Anolyte (1/250) were the most effective disinfectants in killing AIV after 10 minutes in laboratory test and application in poultry house.

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Dust in Riding Halls: A Potential Health Threat to Horses and Men?

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Summary: The aim of this ongoing study is the evaluation of the air quality in riding halls with a special emphasis on particle load and occurrence of bacteria. The longitudinal monthly sampling allows a detailed investigation on the dynamics of microbial and particle load over time before and after a standardized riding program. Four riding halls in Saxony-Anhalt, Germany, with different footing material are visited monthly over the course of one year. The measurements take place at defined points of the riding halls at the height of the horse's and the rider's nose(1. 5 m and 2. 5 m over the ground). The spectrum of analyzed bacteria in combination with the respective dust load provides useful information on the actual exposure and possible health consequences for horses and men.

Introduction

The air quality, especially the dust load, in horse stables has been analyzed in several studies during the last few years. However, available studies on the air quality in riding arenas are more than 20 years old [1,2]. Besides colics, respiratory diseases represent the most common internistic problems in horses[3]. Acute and chronic respiratory diseases, such as RAO(recurrent airway obstruction) or IAD(inflammatory airway disease) are often multifactorial. Predisposing factors for the development of clinical disease are the exposure to dust or contaminants. Although riding horses, and also riders, spend a high percentage of their lifetime in riding halls, knowledge on the occurrence of different fractions of dust and attaching microorganisms in this environment is lacking. During riding, horses stir up dust from the ground, which is expected to influence the air quality. Especially with regard to the horse's high air consumption during training, the air quality in the hall is of essential importance[4]. To determine the potential health risk for horse and rider in riding halls, studies on air quality including measurements of dust particles and bacteria are necessary.

Material and methods

Halls and sampling

In total, sampling is planned over the course of one year. However, in the represented part of this ongoing study, samples were collected monthly over four months in four riding halls located in Saxony-Anhalt. While the riding arenas were comparable in their size(40 m × 20 m), they differed structurally in footing material, age of the ground ordirect proximity to the stable Table 1.

Table 1 Details of the four sampled riding arenas

	Arena I	Arena II	Arena III	Arena IV
Number of riding horses	32	24	50	56
Direct proximity to stable	yes	no	no	yes
Footing	sand	sand/wood shavings	sand	sand
Age of footing material	4 years	2 years	0.5 years	2 years
Underground	natural ground	natural ground	natural ground	natural ground

Air samples as well as ground samples were taken on four arena points(the middle of the short and the long sides) before and after a defined riding program. Air measurements were conducted in two different heights: the horse's and rider's nose(1. 5 m, 2. 5 m).

To investigate the air quality, we focused on dust load and bacteriological analysis. The concentration of dust particles in the air was measured for six fractions (0. 3, 0. 5, 0. 7, 1. 0, 2. 0, 5. 0 μm) using the HHPC-6 Airborne Particle Counter (Argo-Hytos, Kraichtal-Menzingen, Germany). Bacteriological sample collection (1l air) was performed with the MAS-100 Eco Air Sampler (Merck KGaA, Darmstadt, Germany) at the same sampling points and heights. Humidity and temperature were recorded during the particle measurements. In addition, ground samples were taken to

compare the bacterial flora in the ground with the flora in the air samples.

Laboratory methods and statistics

For the bacteriological air sampling, blood agar and selective agar for *Staphylococci* (Bird Parker; Oxoid Deutschland GmbH, Germany) were used. This study focused on *Staphylococci*, because they had been identified as the predominant bacteria family during an initial screening of air samples over the course of 8 weeks before the actual study started. In this pilot study, no gram-negative bacteria were detected. After sampling, agar plates were immediately incubated at 38℃ for 24 h. *Staphylococci* were quantified and biochemically identified using API ID 32 STAPH, BioMérieux SA, Marcy-l'Etoile, France).

Ground samples were diluted (1 g soil/100 ml aqua dest.), streaked out onto blood agar plates and incubated at 38℃ for 24 hours. Subsequently, *Staphylococci* were analyzed according to the air samples. Data analyses and statistics were performed using the statistical analysis software SAS 9.2[5].

Results

The represented results were assessed at the first four months of the ongoing study. Particle concentration of the dust before the riding activity ranged from 60,000 – 120,000 particles/l. In Arena II, the lowest dust loads were analyzed (Fig. 1). After the riding activity, the concentration of dust tended to increase between 65,000 – 135,000 particles/l ($P = 0.1153$). The two different heights had no influence on the particle count ($P = 0.9119$).

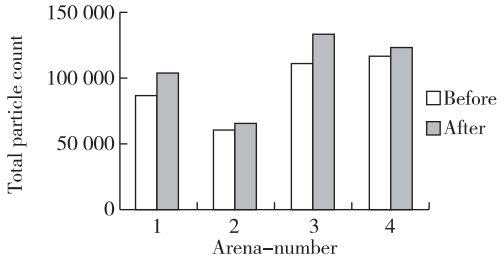

Fig. 1 Dust load in the arenas before and after the riding program

The seasonal decrease of temperature over the study period significantly correlated to the total number (CFU = colony forming units) of *Staphylococci* (Fig. 2) as well as to the total amount of dust particles.

Air samples cultivated on blood agar ($n = 240$) contained 1.53 bacterial strains on average per sample. In 6% of the samples, no *Staphylococci*, in 31% one, in 29% two and in 34% three to five isolates were found. In

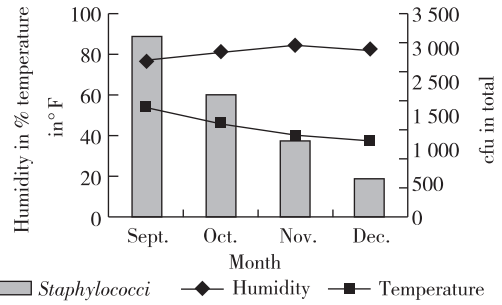

Fig. 2 Changes in the total amount of *Staphylococci* isolated from air samples of four riding halls, relation to measured humidity and temperature in °F

total, 527 *Staphylococci* were isolated, which belonged to 26 different species. *Staphylococcus xylosus* clearly dominated, whereas *S. equorum*, *S. capitis* and others showed minor prevalence.

Table 2 Distribution of *Staphylococci* species in air and ground samples

Bacterial species	Air	Ground
S. xylosus	82%	70%
S. equorum	5%	11%
S. capitis	3%	2%
S. lentus	2%	1%
S. hominis 1	2%	0%
S. intermedius	1%	0%
Others	5%	16%

Ground samples ($n = 120$) showed an average of 1.0 identified bacteria per blood agar plate. There were 30% of samples with no, 54% with one, and 16% with two to four identified *Staphylococci* species. All species found in the ground samples were also detected in the air samples with *S. xylosus* as major species. The total diversity of bacterial species was higher in air samples ($n = 26$) than in ground samples ($n = 9$). There were no significant differences of cfu in the different air samples before and after the riding program.

Discussion

The assessed data in this study clearly indicated a high concentration of respirable dust particles in riding halls. In comparison to the concentration of respirable dust particles of wood shavings as bedding in a horse stable (~ 40,000 particles/l) [6], and good hay (~60,000 particles/l) [7], the concentration in riding arenas was two to three times higher (~ 60,000 – 138,000 particles/l). Between the analyzed riding halls, differences in the dust load were found. Arena II showed only half of the amount of dust particles, and low numbers of bacterial contamination. This might be related to the different footing material with wood shavings in

Arena II, compared to sand in the other halls. The relation between increasing bacteria numbers and temperature and humidity is in agreement with the results of Saleh[8]. nut in contrast to the study of Banhazi[9]. Regarding the detected bacteria, Nagase et al. [10] found an incidence of 23.5% for *S. xylosus* on the skin of horses. In our study, *S. xylosus* was found in 82% of the air and 70% of the ground samples. The pathogenic potential of the dominant *Staphylococci* species isolated in this study remains unknown, because no data exists on their direct effects on lung tissue of horses and men. However, a high dust load and bacterial contamination might be a risk factor if the exposure time is long-term, or individuals are immunocompromised.

Conclusions

Our findings indicate that both, horse and rider, are exposed to contaminated air with respirable dust particles and bacteria in riding areas, especially during riding, via contamination from the ground. To determine the resulting risks in detail, long-term data collection and analyses are necessary. Therefore, further effects on air quality, for instance the occurrence of fungal spores and the loads of dust and microorganisms over the course of one year, will be object to further studies in this project.

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Ways to Decrease Environmental Contaminations of Antimicrobials from Swine Livestock

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Summary: The aim of the present study was the determination of potential ways to avoid environmental contaminations by antibacterials, since their frequent use of antimicrobials in livestock farming poses a risk for the contamination of the environment (especially soil, surface water and dust). In the present study, the effect of different pharmaceutical formulations on the entry of the sulfonamide sulfadiazine (used as test compound) into the environment was investigated in pigs.

Six pigs were orally treated with two formulations of sulfadiazine (25 mg/kg; powder vs. pellets) over four days to obtain information about the effect of different formulations on the environmental contamination. To study a carry over via the environment, the treated pigs were replaced by non-treated pigs on day 5. Thereafter, blood and urine samples of the pigs and samples of sedimented dust in the stable were analysed for sulfadiazine, as well as air filters installed apart from the housing compartment. Therefore, UV/VIS-High performance liquid chromatography was used.

Powder and pellet feeding result in comparable plasma and urine levels, but massive differences in the environmental pollution. Pellets decrease the entry of sulfadiazine into the stable. Non-treated pigs housing in a contaminated stable after powder treatment of sulfadiazine exhibit traces of sulfadiazine in plasma and urine.

Using pharmaceutical formulations like pellets, the environmental pollution of sulfonamides can be diminished significantly compared with powder due to a massive dust reduction during the feeding process. Nevertheless, carry over processes have to be considered.

Introduction

In the antibacterial treatment of animals in Europe, tetracyclines, β-lactam antibiotics and sulfonamides are the most frequently used antibacterial agents. Sulfonamides have a wide antibacterial action against gram-positive and gram-negative bacteria and they also have activity against some rickettsia and protozoa [1]. The chemical structure of sulfonamides is characterised by a benzene ring in combination with a sulfanilamide group [2]. The antimicrobial activity of sulfonamides is caused by the para position of the substitutes. Derivatives differ in the substitutes bound to the nitrogen in the sulfonamide group and hence show differences in metabolism, solubility, protein binding and excretion [3 – 6].

After therapeutic treatment most sulfonamides are only partially metabolised in the organism and, hence, pose a risk as environmental pollutants due to the excretion via urine or faeces [6 – 9]. Remaining antimicrobial activity in some of the metabolised products [10] consequently might also entail a risk of the development of antibacterial resistance. Contamination from animal husbandry primarily affects soil and water [8, 11, 12]. Furthermore, sulfonamides contaminate water in particular by surface run off, sediment of shrimp ponds [13 – 15] and sulfamethazine leaching from soil has been detected in groundwater [16].

The main problem of antibiotic residues in the environment is the development of bacterial resistance due to concentrations beyond the required concentration to assert a definable effect in the microorganisms [17]. Antibiotic residues in soil can be incorporated by plants after distribution of manure onto the acreage of various agricultural crops, as already shown for tetracyclines and sulfadiazine [20, 21]. Thus, the uptake of contaminated plants with antibiotics in subtherapeutic concentrations may be another health risk for animals and humans. Furthermore, insects in the animal production environment may furthermore play an important role in the dissemination of antibiotic resistant bacteria as recently demonstrated [22].

Therefore, the present study investigates the possibility to reduce environmental pollutions of sulfadiazine as test substance after antibiotic treatment of pigs.

Material and methods

Animals

18 female piglets (10 – 15 kg bodyweight) of a hybrid breed, weighing 10 – 15 kg at the start of the

experiment, were obtained from the "Lehr- und Forschungsgut Ruthe". They were kept in groups of 6 animals and had free access to tap water from drinking nipples. All pigs were kept under the same conditions. The whole stable had 23.52 m^2, with 6 animals in bays of 8.8 m^2. A ventilation system maintained 19 – 20℃ in the stable.

All animals were clinically healthy during the entire experiments. The experiment was approved by the ethical committee of the University of Veterinary Medicine Hannover, Foundation/LAVES.

Experimental procedure

Pharmacokinetic studies were carried out in three groups of 6 pigs to determine the oral availability of sulfadiazine and the environmental pollution of treated pigs. Therefore, sulfadiazine was supplemented to the feed (powder, pellet), which was given twice a day. Blood was sampled into heparinised tubes immediately before feeding and after 3 hours. Urine samples were taken spontaneously once a day.

The first group of 6 pigs was treated via powder feeding as follows: After 1 week of acclimatisation, the pigs were weight and received different concentrations of sulfadiazine (Trimosulf, WDT, Garbsen, Germany) in powder over 4 days in order to stimulate environmental pollution and treatment according to the package leaflet. Between each treatment a washout-phase of three days was inserted. The first treatment was 2.5 mg/kg BW (body weight) for 4 days, followed by a washout-phase of three days. Afterwards, each pig received 5.0 mg/kg BW sulfadiazine for 4 days, followed by three non-medicated days. Finally, 25 mg/kg BW sulfadiazine was orally administered to the pigs (concentration according to the package leaflet) for four days. Afterwards, the pigs were removed from the stable, which underwent a dry cleaning process and 6 new piglets moved into the stable in order to stimulate a carryover of sulfadiazine of a polluted stable. These untreated pigs were kept in the stable for 6 days and were fed with powder feed without antibiotics.

The second group of pigs was treated with manufactured formulations of sulfadiazine (pellets) in order to determine environment-friendly treatment formulations of sulfadiazine. This group was treated with sulfadazine (25 mg/kg BW) via pellets over 4 days. The pellets were generously manufactured by the Institute of Animal Nutrition, University of Veterinary Medicine Hannover, Foundation.

During the entire experiment, sedimentation dust samples were taken from five different localisations in the stable. Furthermore, two air filters were installed to filter sulfadiazine from the stable air.

A modified extraction protocol was used to extract sulfadiazine from all samples [23]. Analysis was performed via high performance liquid chromatography.

Results and discussion

All dosages of sulfadiazine given via powder resulted in measurable mean plasma concentrations 0.4 – 1.0 μg/ml (2.5 mg/kg = 10% of the recommended dosage), 0.8 – 2.0 μg/ml (5 mg/kg = 20% of the recommended dosage) and 5.2 – 14.2 μg/ml for the recommended dosage (25 mg/kg). Untreated pigs living in the stable after removal of treated pigs exhibited plasma concentrations of 0.05 – 0.08 μg/ml over 4 days.

Urine samples of powder treated animals exhibited sulfadiazine concentrations of 7.7 – 21.5 μg/ml (2.5 mg/kg), 23.8 – 45.0 μg/ml (5 mg/kg) and 112.3 – 311.5 μg/ml (25 mg/kg) over 4 days, while untreated animals exposed to the treatment stable showed declining urine concentrations (3.5 μg/ml to 1 μg/ml) over 6 days.

Pellet feeding of 25 mg/kg sulfadiazine resulted in comparable plasma and urine levels (4.6 – 11.4 μg/ml plasma, 83.5 – 140 μg/ml urine) to powder feeding.

During the powder feeding period sulfadiazine was detectable in sedimented stable dust in five localisations in concentrations increasing with higher dosages with concentrations of up to 2.7 μg/mg dust. Aerosol analysis revealed sulfadiazine concentrations up to 8 μg/m^3 air during the treatment with the recommended dosage (25 mg/kg).

Animal treatment with sulfadiazine via pellets massively declines the sulfadiazine concentrations in sedimentation dust and aerosol (Fig. 1) in comparison to powder feeding.

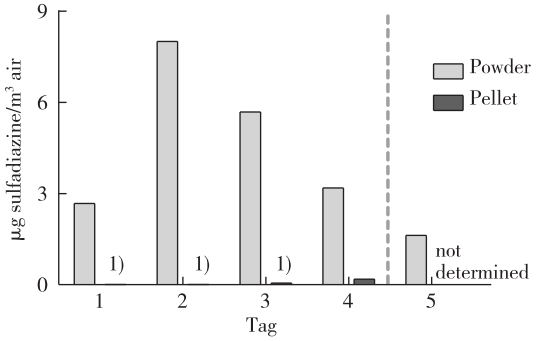

Fig. 1 Sulfadiazine concentration in aerosol of the stable of 6 pigs treated with sulfadiazine (25 mg/kg) via powder (light grey) or pellets (dark grey) for 4 days

Conclusions

Using pharmaceutical formulations like pellets, the environmental pollution of sulfonamides can be

diminished significantly compared with powder due to a massive dust reduction during the feeding process. Nevertheless, carry over processes have to be considered.

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Nanotechnology and Poultry Production

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Summary: Nanotechnology is the manipulation or self-assembly of individual atoms, molecules, or molecular clusters into structures to create materials and devices with new or vastly different properties. Nanotechnology, defined as that technology in which materials and structures are found in nanometric scales, has increased his application and research, since small scales materials have different properties and characteristics compared to those at higher scale. Nanotechnology holds promise for medication and nutrition because materials at the nanometer dimension exhibit novel properties different from those of both the isolated atom and bulk material. Recently, research on nanobiotechnology has been increased, especially that focused on drugs for human health, and there is the need of applying that knowledge on animal health, in order to improve poultry production process. However, there is a lack or null information about it, even though research focused on human health is carried out using animal models. It is important to take into consideration that nanotechnology must include ethical, environmental and food safety factors. Therefore, the objective of this review is to offer a general view on nanotechnology and its application on veterinary and poultry production, based on the new challenges to explore new research areas according to actual needs to obtain benefits for the society.

Introduction

The definition of nanotechnology is based on the prefix "nano" which is from the Greek word meaning "dwarf". In more technical terms, the word "nano" means 10^{-9}, or one billionth of something. For comparison, a virus is roughly 100 nanometers (nm) in size. The word nanotechnology is generally used when referring to materials with the size of 0.1 to 100 nanometers, however it is also inherent that these materials should display different properties from bulk (or micrometric and larger) materials as a result of their size. These differences include physical strength, chemical reactivity, electrical conductance, magnetism and optical effects. [4]. This implies a certain understanding and control of matter measuring from 1 to 100 nm, with the capability of having new applications. This is because at sizes so small, the properties of matter can differ considerably from those at larger scale, even in microns. For a better understanding, a nanometer is a billionth of a meter (10^{-9}) and, as reference, the thickness of a sheet of paper measures approximately 100,000 nm [15], and the length of a visible light wave ranges from 400 to 700 nm; also, many biological structures are sized at a few nanometers (Table 1) [21].

Worldwide, financing for research in this area comes from both public and private sources. The United States of America, Japan, and Germany are the countries that have the greatest public investment in Nanotechnology (1,606; 1,100; and 413 million dollars, respectively, in 2005) [16]. No Latin American country appears in the list of the 13 countries with the highest budget for nanotechnology studies within that year.

Table 1 Size of different biological structures (nm)

Biological structure	Size, nm
Leucocytes	10,000
Bacteria	1,000 – 10,000
Virus	75 – 100
Protein	5 – 50
DNA (width)	– 2
Atom	– 0.1

Historically speaking, nanoparticles have existed on the planet for a very long period of time, and they have been created though several natural phenomena, including photochemical reactions, volcanic eruptions, or forest fires [4]; and, although from recent research, the applications of nanotechnology have been present since ancient times, even if in those days they did not refer to it as such. It is known, for example, that the pigment used for porcelain in the different Chinese Dynasties (since the XVI thcentury B. C.) contains nanoparticles of gold [12]. Another case is that of the Lycurgus cup (on exhibition at the British Museum) made during the Roman Empire (IVth century B. C.), which possesses a glass matrix containing nanoparticles of gold and silver, responsible for creating an optical effect in the cup depending on the direction of the light: if the cup reflects the light, it looks green, if it transmits it, it then changes

to red [17]. In the Orient, colloids containing nanoparticles of gold were also used in the treatment of bone ailments such as arthritis [6]. Later, in the middle of the XX thcentury, the physicist [10] proposed the possibility of handling matter atom per atom, leaving the door open for the development of a technology at atomic and molecular scale. In 1975, Ringdorf developed a joint pharmaco-polymer model, stressing that the properties of said model can change depending on the properties of the polymer [22]. The applications of nanotechnology are indeed very varied. For example, one of the most important and extensive uses of nanotechnology is in the area of human medicine, where it has allowed the development of nanoparticles for the controlled liberation of cancer medication [22], nutrients [19], hormones [20], gene therapy [2], and as a contrast medium for image studies [14], among others. In the area of veterinary medicine and animal production, there is a growing interest in the application of nanotechnology in its processes. However, research in this field is still very limited[17].

Differences between nanomaterials and larger materials

The physical, chemical, electrical, optical, mechanical, and magnetic properties(as well as others, still unknown) at an atomic scale are quite different from those present at a greater scale, even when compared with those present at a scale of microns(10^{-6}) [4,6]. The reason that nanomaterials are so different from larger ones is, according to Roduner, because of two effects:

Surface. The atoms of nanomaterials are less stable than those of larger structures since the energy required to join adjacent atoms is less. As a consequence of this, the fusion point of a given element changes.

Quantum effects. Quantum points are a type of nanostructures, just a few nanometers in size, that show a behavior similar to a single atom. Their spatial arrangement allows them to have properties not proper to the element, such as magnetism in metals like gold or platinum when they are in the form of nanoparticles.

Moreover, nanoparticles have a surface area much larger than microparticles. To illustrate this point, a carbon microparticle with a diameter of 60 m has a mass of 0.3 g, and a surface area of 0.01 mm^2; the same mass of carbon forms 1 trillion particles, 60 nm in diameter, with a surface area of 11.3 mm^2. This indicates that as the size of the nanoparticles decreases, the surface area for chemical reactions increases, thus reactivity increases about 1,000 times [4]. The aforementioned can be compared with the function of cilia and micro-cilia of the intestinal tract, present in every animal species, including man. The intestinal tract is covered with epithelial projections called cilia, which increase the surface area 10 to 14 times more than if it were a flat surface. Also, these cilia are covered with microscopic micro-cilia that increase even more the total surface area [8].

Preparation and design of microparticles

There are different methods for the preparation of nanoparticles. The selection of any of these methods depends on the particular objectives and conditions for where and how the obtained particles are meant to be used. Thus, it is necessary to consider the physical and chemical stability of the active agent, as well as its toxicity, its liberation profile, among many other considerations. [1] specifies some common methods for the preparation of nanoparticles, such as:

1. Cross-linking emulsion: in this method, a water-oil(w/o) emulsion is prepared through emulsification of a watery solution in an oily phase, which when shaken vigorously separates and hardens the particles. It requires the use of agents that facilitate the union of the involved agents.

2. Precipitation/coacervation: in this case, the particles are produced by "blowing" the interest agent in an alkaline solution. The separation and purification of the particles is done through filtration and centrifugation, followed by rinsing with hot and cold water.

3. Spray-Drying: this is one of the best-known techniques used to produce dusts, granules, or agglomerates, besides being an easy and quick way to do it. It is based on the drying of droplets sprayed into compressed hot air. It requires the use of a solvent(for example, a solution of acetic acid), which is instantly evaporated, allowing the formation of particles.

Another important factor to take into account is the shape that the nanoparticles acquire, since it strongly influences its biological behavior. It is important to point out that these particles are not always spherical, as could be expected. There are reports of innumerable shapes, some quite peculiar, of nanoparticles: rectangular discs, cones, canes, "worms", elliptical or circular discs, "rolls", among many others. All these can come up in the 1st, 2nd, or 3rd dimension, depending on the preparation method and the materials used. Concerning this, the viscosity and thickness of the material used determines whether the particle will show sharp or flattened endings. It is even possible that the nanoparticles will show regions with different curvature, texture, concavity, and other characteristics [7].

Besides capsules, other nanostructured materials can be used, which have the potential of changing the structures of other particles. Some specific examples of these are fullerenes(structures made up of 60 – 80 carbon atoms arranged in spherical shapes, used for the controlled liberation of medication), dendrimers (branched structures which, due to their structure, can

serve as vehicles for medication, liberating it in a specific location), and quantum dots (nanometric crystals designed for optical and electronic applications. When a quantum dot is stimulated, it emits a fluorescence of varying intensity) [17].

Possible applications in animal production

A great portion of nanotechnology research applied to human medicine has been tested in lab animals (rats, mice, rabbits, broiler, goats, guinea pigs, among other species), and thus these nanotechnological applications are, indeed, susceptible of being studied in species of a zootechnical interest, wild fauna, or pets. It is probable, however, that there lacks a greater interdisciplinary involvement among professionals of biotechnology (including nanotechnologists, of course), veterinary science, zootechnics, agronomy, and akin areas. Regarding this, [21] points out four possible applications of nanotechnology in animals: 1) administration of medication, nutrients, probiotics, supplements, and other substances, 2) diagnosis and treatment of diseases with nanoparticles that allow the detection and elimination of the cause of the disease without the need for surgery, 3) identity registry that allows a follow up on the history of an animal and its products (meat, milk, eggs, mainly), and 4) management of reproduction with hormonal immunosensors.

Actually, there is already research aimed at animals of zootechnical interest, although the number of studies is still quite reduced. Recently, [18] designed and evaluated, *in vitro*, sodium selenite nanoparticles for oral use in ruminants using copolymers of metacrylate, sensible to pH, such that they would not be degraded in the rumen (near neutral pH), but would in the abomasums, whose pH is acid due to the secretion of chlorhydric acid, similar to that present in non-ruminant species; however, no *in vivo* tests were done.

On the other hand, the routine use of antibiotics in animal production systems is a given fact, and these can leave a residue in the products that reach the final consumer. And although there is a variable retirement period before the products of treated animals can be placed into the market, this period is not always respected. However, with the use of nanotechnology, the amount of antibiotics used can be greatly reduced due to the properties that the substances acquire when their size is reduced to a few nanometers. This was proven by [9] on mice infected with *Salmonella typhimurium*, and treated with different forms and amounts of ampicillin. Those treated with ampicillin joined to nanoparticles had a survival ratio equal to those treated normally; the difference being that the prior required 40 times less antibiotic to achieve the same effect. Moreover, its distribution in the tissues was more specific, thus validating the lower amount of antibiotic required to equal the results. In the area of nutrition, it is also possible to apply nanotechnology with several goals, such as obtaining information of a nutrient or bioactive component, its liberation in specific sites of action, greater availability, maintenance of adequate levels for longer periods of time, avoiding its degradation, and lower parenteral invasion [19], thus also reducing the stress implied in animal handling. Minerals are one of the most widely used supplements in animal nutrition; however, the way in which said minerals are found influences their bioavailability, and so, if they have low bioavailability, the animal will not make suitable use of them, and they will be eliminated.

Applications in poultry production

Silver nanoparticles were tested unsuccessfully (with antibacterial aims) in chicken embryos. The particles, although they did not affect embryo development, reduced the number and size of lymph follicles of the bursa of Fabricius [11]. Also, [23] demonstrated that effects of nanoparticle paprika oleoresin (1 and 3 g/100 mL) on the physical and sensory properties of cooked marinated chicken, and the results cleared that marinating performance and sensory acceptability of marinated meat products can be improved and optimized by the utilization of nanoparticle ingredients in marinating operations.

In the experiments carried out by [24] the effect of feed supplementation with nano elemental Se on Guangxi Yellow chicken. Their results revealed that that supplementing diets with 0.30 mg/kg of Nano-Se for was effective in increasing the growth performance and feed conversion ratios of chickens, the Se content of tissues, and the quality of the meat. In addition, [13] reported that feeding (Nano-Se) in Arbor Acre male broiler chickens as compared with sodium selenite, showed that the range between optimal and toxic dietary levels of Nano-Se was wider than that of sodium selenite, and Nano-Se was more efficiently retained in the body than sodium selenite. Moreover, According to [5] broiler chicks received diets supplemented with 0.0, 0.3, 0.5, 1.0, or 2.0 mg/kg of (nano-Se) had significant effect in glutathione peroxidase activity, free radical inhibition, contents of IgM, glutathione, and malondialdehyde in serum, on glutathione peroxidase activity, free radical inhibition in liver, and on glutathione peroxidase activity in muscle, with birds fed 0.30 mg/kg of nano-Se exhibiting the best effect and birds fed 2.0 mg/kg of nano-Se showing the worst effect on these parameters.

Safety and toxicity of nanomaterials used in animals

It is important to determine what is the zootechnical use of the species that will be given the nanoparticled component. It is not the same if it is one which will be used as a pet or for exhibition, as in the case of zoo

animals, or if it will be used, the animal itself or its products, for human consumption, due to the risks of residues in the tissues. Generally speaking, the toxicity of a nanoparticle depends on several factors, such as dose, the concentration of the interest agent, the type of polymer used to nanoencapsulate, among others. Regarding this, [3] found that nanoparticles of silver negatively affect gametogenesis in mice, and therefore this element should be avoided when using animals destined for reproduction.

Conclusions

Nanotechnology is in constant development, and its applications are ever more varied and specific, with a high potential for improving livestock production, and animals in general. The study of nanotechnology in these areas is still very limited; regardless, it is feasible to apply it, probably with encouraging results that will allow to carry out processes more quickly and efficiently and, perhaps, at a lower risk to consumers. However, a great amount of research is still required to support the effectiveness, and mainly the safety of nanotechnology, avoiding any harm to the environment or to human beings proper.

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Efficiency of Two Air Sampler for Determination of Mesophilic Bacteria in Laying Hen Flock

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Summary: The air in laying hen houses contains high concentrations of airborne bacteria. The numbers of these bacteria can be influenced by the efficiency of the chosen sampling method. In this study we compared AGI-30 Impingers and the Coriolis®μ air Sampler in terms of their efficiency of sampling aerobic mesophilic bacteria in a laying hen house for 6 week during of the laying period. The numbers of mesophilic airborne bacteria ranged from 8×10^4 to 2×10^6 CFU/m^{-3} when sampled using AGI-30 Impingers and from 2×10^5 to 4×10^6 CFU/m^{-3} when sampled using the Coriolis®μ air Sampler. The concentrations detected simultaneously by both devices correlated well ($r_{Pearson} = 0.755$); but the Coriolis®μ air Sampler showed a significantly higher sampling efficiency ($P < 0.001$). The Coriolis®μ promises to be a useful technique to quantify aerobic mesophilic bacteria in poultry houses efficiently.

Introduction

A standardized method, which was used several times to measure concentrations of airborne culturable bacteria in poultry buildings, is the impingement with AGI-30 impingers [1]. This technique samples airborne microorganisms in a fluid (usually buffer or water) and allows detecting high microbial concentrations in animal house air [2]. Using AGI-30 impingers in poultry houses may also deliver comparable results to former studies [3]. Thus, we used a novel air sampler that recently showed a low detection limit for airborne bacteria cells Coriolis®μ [4] and took the AGI-30 impinger as a reference method to compare the sampling efficiencies of the devices. Air samples were tested for the total number of culturable mesophilic bacteria.

Materials and methods

Sampling locations and sampling period

Samples were taken in a forced ventilated laying hen house equipped with an aviary system (NATURA 60, Big Dutchman, Germany). Two thousand three hundred laying hens (breeding line "Silver", Lohmann, Cuxhaven, Germany) were kept in this multilevel system with nest boxes at the sidewalls and a littered scratching area inside. Samplings were done weekly between 10.00 am to 13.00 pm, beginning on the 14th and ending on the 19th week of one laying period. Air samples were taken lengthwise in the centre of each third of the laying hen house. The instruments were placed 1.5 m above the scratching area. Temperature and relative humidity (RH) were measured at the same height in the mid-position with a thermo-hygrometer (Rotronic Date logger Hydrolog-D HygroClipS Temperatur/RH, Rotronic GmbH, Ettlingen, Germany).

Sampling of airborne bacteria

The impingement with all-glass impingers (AGI-30; Ace Glass Inc., Vineland, NJ, USA) and a wet cyclone technology (Coriolis®μ Air Sampler, Bertin technologies, Montigny le Bretonneux, France) were used to sample airborne bacteria. At each sampling day, three impingers were operated simultaneously at the sampling locations for 30 min. Micro-organisms were collected in 30 ml phosphate buffered saline (PBS). The air flow (12.5 l·min^{-1}) through the impingers was controlled before and after the end of the sampling time with a flow meter 044-14G from Analyt-MTC (Mülheim, Germany). In order to compare the impingement with the Coriolis® μ Air Sampler, one air sample was taken with the cyclone at each sampling position in parallel to the impingement. The cyclone was adjusted to sample 0.9 m^3 within 3 min. Airborne bacteria were collected in Coriolis®μ cones filled with 15 ml PBS.

At each sampling day, one transport control for each sampling methods, were carried along with the air sampling. All samples were analysed on the same day.

Laboratory analysis of air samples

Impingers and Coriolis®μ cones were shaken for 30 s at full speed with a Vortex-Genie2 (Scientific Industries Inc., USA) and 1 ml aliquots were taken from the sampling solutions to prepare serial dilutions (10^{-1} to 10^{-4}). Three times aliquots (0.1 ml) from the original

sampling solution and from the dilutions were plated on blood agar base (Oxoid, Germany). The plates were incubated aerobically for 48 h at 36℃. Subsequently, the average numbers of colony forming units (cfu) of one dilution step with countable colonies (between 30 and 300 colonies per plate) were used for calculating the total culturable airborne bacteria per cubic metre [c] by the following equation:

$$c = \frac{cfu}{V_{plated\ aliquote}[\mathrm{ml}]} \times \frac{dilution\ factor \times V_{buffer\ after\ sampling}[\mathrm{ml}]}{V_{air\ sample}[\mathrm{m^3}]} \quad (1)$$

Statical analysis

Statistical differences among the numbers of bacteria detected with the impingement and the Coriolis®μ Air Sampler were assessed by using the Wilcoxon sum-rank test. The correlation (Pearson's correlation) among the bacteria concentrations measured with different sampling techniques was calculated with the SAS [5] software version 9.3 [SAS Institute Inc., Cary, NC, USA].

Results

The concentrations of airborne mesophilic bacteria from impinger samples ranged from 8×10^4 to 2×10^6 CFU/m^{-3} and the concentrations from the Coriolis®μ Air Sampler varied between 2×10^5 and 4×10^6 CFU/m^{-3}. No bacteria growth was observed in any of the transport controls. In 17 out of 18 air samples the Coriolis®μ Air Sampler showed higher bacteria concentrations than the AGI-30 samplers (Fig. 1). The differences between the concentrations of both air samplers are highly significant ($P < 0.001$). The tendencies of the concentrations of the different sampling methods are very similar and show a high correlation ($r_{Pearson} = 0.755$). There was no obvious coherence between climatic factors and the average bacteria concentrations of both the sampling methods (Table 1). In this context, it is remarkable that the maximum variations of temperatures (±3.6℃) and humidity (±17%) were low among the different sampling days.

Table 1 Amounts of mesophilic bacteria × 10^4 CFU/m^{-3} (Mean ± SD) at different ages of laying hens and under different climatic conditions in the laying hen house

Sampling no. (week of laying period)	Air sampling methods		Temprature (℃)	RH (%)
	Impinger	Coriolis		
1(14)	145 ± 71	158 ± 38	16.8	45.5
2(15)	32 ± 22	52 ± 22	13.2	32.6
3(16)	37 ± 18	60 ± 41	15.8	49.6
4(17)	25 ± 24	50 ± 28	13.9	46.6
5(18)	34 ± 6	58 ± 28	13.2	43.2
6(19)	87 ± 32	233 ± 130	14.6	46.4

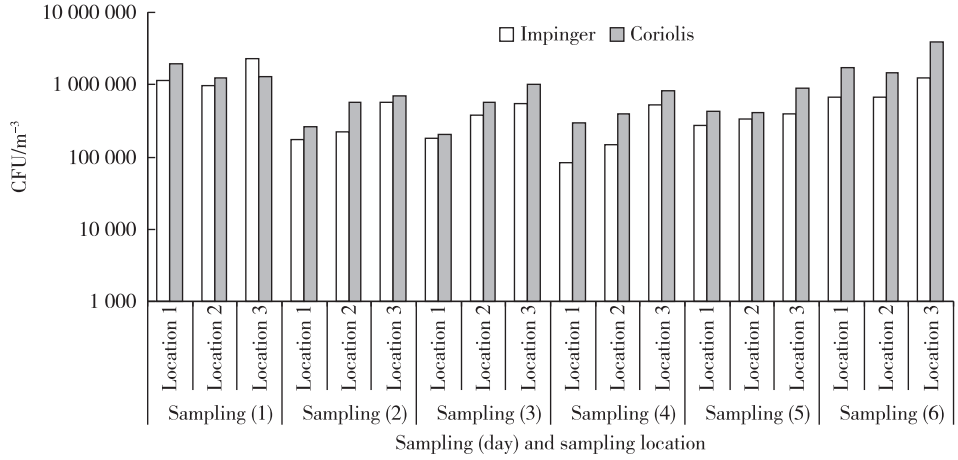

Fig. 1 Airborne bacterial concentrations (CFU/m^{-3}) detected simultaneously with Impingers and Coriolis®μ Air Sampler on 6 different sampling days

Discussion

Measurements with both applied air-sampling techniques showed a strong variation of bacteria concentrations (> one log step) between the 14th and 19th week of the laying period. Such different values of airborne bacteria were also detected with AGI-30 impingers in aviaries in a seasonal course by Springorum and Hartung [6]. The authors suggested that air exchange rates, the animal activity and the waste management are important factors having an impact on the concentrations of airborne microorganisms in a laying hen house. We assume that these factors have also affected the bacteria concentrations during our investigations. Interestingly, a high correlation was observed between the bacteria concentrations detected with different sampling methods. This indicates that probably the same factors (animal activity, ventilation rate etc.) within the laying

hen house have influenced the results. However, the concentrations measured with the Coriolis®μ Air Sampler were significantly higher than the concentrations detected with AGI-30 impingers. One reason for that could be the difference in particle sizes sampled by them from the air. The Coriolis®μ Air Sampler samples larger particles compared to the AGI-30 impinger. These larger particles may carry more bacteria than smaller particles [7] which could lead to a higher bacteria concentration in the sampling buffer of the Coriolis®μ cones. Other reasons could be the sampling stress induced by the impingement and a minor loss through reaerosolization of particles within the cyclone [8]. The reasons for these differences need to be clarified under laboratory conditions in future. However, due to the higher sampling efficiency of culturable bacteria and its lower detection limit compared to the AGI-30 impinger, the Coriolis®μ Air Sampler seems to be a suitable device to measure bacteria concentrations in the air of animal houses. It is recommended to verify this statement by conducting further experiments in different housing system. Also a potential impact of climatic conditions (temperature and relative humidity), which showed no obvious influence during our experiments, should be examined in more detail.

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Microbial Environmental Contamination and Zoonotic Risk of Antibiotic Resistant *Escherichia coli* in Broiler Chickens

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Introduction

Hygiene of housing is an important factor to be considered in intensive poultry breeding. The air in animal facilities can be a reservoir of primary and potentially pathogenic micro-organisms involved in the etiology of infectious diseases. Air quality in a broiler house is determined by a complex interaction between many factors including ventilation, stocking rate, litter quality and health status of the birds.

The aim of the study was to compare the occurrence of ESBLs among environmental *E. coli* isolates from dust and air and rectal *E. coli* isolates from chickens.

Although, the European Union(EU) banned the use of antimicrobial drugs for growth promotion (Regulation EC, No. 2821/98) antibiotics are widely used in modern livestock and poultry production to treat sick animals. Moreover, they are also administered at subtherapeutic doses, usually in water or feed, to protect animals against disease.

Nevertheless, since this ban increasing quantities of antimicrobial agents have been used for therapeutic purposes by veterinarians. From among these agents tetracyclines, quinolones, and sulfonamides are antimicrobial families that belong among the most widely used in poultry therapy [4].

Material and methods

Monitoring of air and dust was performed on an intensive broiler farm from first day of fattening up to the slaughter. In regard to the fact that the broilers are very sensitive animals, regular examination of microclimate parameters such as temperature, humidity and air velocity are very important to ensure animal welfare.

The broilers were kept on 10 cm deep litter(sawdust and wood shavings). The observed broiler house was a single storey houses. There were 13000 chickens stocked in the area of approximately 1100 m^2 which complied with the requirement on stocking density (30 kg/m^2). Temperature and relative humidity was monitored with a mini datalogger Testo 174H and dust concentrations was measured with Microdust Pro Casela.

Bioaerosols were collected by means of a sampler MAS-100 Eco. The MAS-100 Eco air monitoring system is a compact sampler intended for use with standard Petri dishes with nutrient media (Meat-pepton agar, Endo agar, Sabouraud agar, MacConkey agar, Uriselect agar). After incubation, the number of colony units was recalculated per 1 m^3 of air.

Twenty one strains of *E. coli* isolates from air and dust and 68 fecal *E. coli* were analysed for the presence of ESBLs by intepretative readings of minimal inhibitory concentrations (MIC) of ceftazidime, ceftazidime with clavulanic acid, ceftriaxon [1] and veterinary ceftiofur and cefquinome. Phenotype interpretation of chromosomal quinolone-resistance mechanisms was done according to Lee et al. [3] with modification according Kmet, Kmetova [2]: low level of MIC for CIP (0.1 – 0.5 mg/L) and ENR (1 – 4 mg/L) represented a single mutation; intermediary MIC for CIP (1 – 2 mg/L) and ENR(4 – 8 mg/L) indicated two mutations(one gyrA and one parC) and high-level resistance MIC for CIP ($\geqslant$4 mg/L) and ENR ($\geqslant$ 16 mg/L) represented three mutations(two in gyrA and one in parC).

Results and Discussion

High temperature, humidity, and particulate pollution levels in poultry buildings support the growth and development of micro-organisms. Total bacterial count in the air ranged from 1×10^5 to $>10^6$ CFU/m^3, coliform bacteria from 1.6×10^4 to $>10^6$ CFU/m^3 and fungi from 1.7×10^4 to 3.95×10^5 CFU/m^3. Average dust concentration was 1.789 mg/m^3 and the maximal value was 7.52 mg/m^3. We found that during fattening of broilers the air contamination increased while dust concentration and fungi remained constant. The predominant genera were *Staphylococcus xylosus*, *Staphylococcus saprophyticus*, *Staphylococcus equorum*, *Enterococcus casseliflavus*, *Staphylococcus aureus*, *Staphylococcus arlettae*, *Macrococcus caseolyticus* and *Escherichia coli*.

An increase in extended spectrum of betalactamase (ESBL) producing *E. coli* strains was observed among bacteria isolated from broiler chickens in recent years (Slovak Antimicrobial Veterinary Resistance database http://www.saske.sk/atbres).

The antibiotics used for prophylaxis and therapy of poultry are often closely related to antibiotics used in human medicine. The classes used include: β-lactams (penicillins and cephalosporins); sulphonamides with and without trimethoprim; tetracyclines; macrolides, lincosamides and streptogramins; and quinolones (including fluoroquinolones). These have a variety of therapeutic and preventive applications in food animals [5].

Table 1 Antibiotic resistance in *Escherichia coli* isolated from broiler rectum (R) and (A) air or dust (number of resistant/total *E. coli*)

Sample	Amp	A + IB	CFF	CFQ	STM	NEO	ENR	TTC	CMF	FLO	COT
Rectal	58/68	1/68	25/68	13/68	24/68	3/68	57/68	46/68	3/68	19/68	24/68
Air	19/21	0	7/21	3/31	10/21	8/21	18/21	15/21	7/21	2/21	8/21

The principal source of betalactam resistance of *E. coli* in poultry keeping is their intestinal microflora. The one e-day old chickens are very sensitive to external environmental influences and to stress factors during their transportation. It is likely that cephalosporins are administered in water already in the first days of life of chickens to reduce their mortality and morbidity. Already the droppings of one-week old chickens contained *E. coli* resistant to cephalosporins (ceftiofur and cefquinome) which gradually decreased during their growth (Table 1). In the fifth week, when the selection pressure of antibiotics subsides, we observed appearance of susceptible *E. coli*.

Table 2 The MIC intepretative readings of the mechanisms of betalactam and quinolone resistance in *Escherichia coli* isolated from broiler rectum and air

Sample	Betalactamases			Quinolones			
	Low	High	ESBL	Multiresistance	Sing	Incom	Full
Rectal	19/68	1/68	24/68 (35%)	8/68	2/68	1/68	53/68 (78%)
Air	5/21	1/21	6/21 (19%)	5/21	2/21	0	18/21 (85%)

Abbrevations. Low- low level of betalactamases, High- high level of betalactamases, ESBL-extended spectrum of betalactamases, Multi-multiresistance, Quinolones: Single- one mutation in gyrA or parC in QRDR, Incompl-two mutations in gyrA or parC, Full-three mutations in gyrA or parC.

The ESBL phenotypes were present more frequently in fecal *E. coli* (24 ESBL positive phenotypes) than in environmental *E. coli* (only 6 positive phenotypes). However, the high level of MICs for enrofloxacin was similar in faecal and environmental isolates (Table 2).

Conclusions

The principal source of ESBL producing *E. coli* are the feces of small chickens. The increase in the resistance of fecal *E. coli* to cephalosporins seems to be caused by the widespread use of these antimicrobials during the first days of chicken life. Environmental *E. coli* were resistant particularly to high level of fluoroquinolones.

Van den Bogaard et al. [6] observed similar pattern of resistance in broiler and turkey farmers and slaughterers corresponded with resistance in animal *E. coli* strain, which proved that identical clones were present in humans and in poultry. The most frequent pattern of resistance in *E. coli* isolates was resistance to streptomycin and sulphamethoxazole. The results in this study strongly suggest a spread of antibiotic-resistant *E. coli* from animals to people-not only to farmers but also at a lower level to the consumers of poultry meats, and hence the low incidence of fluoroquinolone resistant *E. coli* in Dutch human population.

Intensive broiler production is a very significant source of air contamination and may pose a health risk tor poultry stockmen and people living in proximity of poultry farms. To prevent the spreading of diseases it is very important to remove the litter and disinfect the entire hall after every turn.

Acknowledgements

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Investigations on the Validation of an Aerosol Disinfection Technique

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Summary: Within these measurements aerosol disinfection is limited to some special applications, e. g. disinfection of aircrafts and the interior of transport vehicles.

Due to the fact that no test methodology is defined so far for the examination of aerosol disinfection in animal husbandry we established such a method in cooperation with DLG and a private company. For this, a combination of a specific apparatus and a specific disinfectant was proved.

A panel of 12 test points was fixed within the test room. At these places stainless steel test bodies holders were placed which carried up to ten single stainless steel standard test bodies coated with the defined test microorganisms (*Enterococcus hirae*, *Escherichia coli*) with and without protein pollution. Aerosol disinfection was performed using an apparatus generating cold fog and a disinfectant based on peracetic acid.

All experiments proved that both the amount and the concentration of the disinfectant plays an important role for the reduction rate of the bacteria and therefore, for the success of the disinfection. In summary, approx. 34 ml of disinfectant per m^3 of the room volume and a 10%-concentration of the disinfectant are adequate to reduce the used test microorganisms for at least four log steps indicating a successful disinfection.

Introduction

The significance of disinfection for prophylaxis against diseases and to fight against epidemics increases in regard to the increasing flock size (DVG, 2007). In Germany there are regulations which have to be fulfilled in the case of an epidemic and which order the detailed steps of disinfection, i. e. chemical and physical disinfection procedures, appropriate biocides, and the performance of the disinfection.

Aerosol disinfection is a simple and elegant solution. Nevertheless, its practical efficacy in the case of epidemic agents is not fully proved so far.

Therefore, the microbicidal activity of aerosol disinfection using a combination of a proved biocide and a specific aerosilisation apparatus was tested in this study.

Material and methods

A panel of 12 test points was fixed within the test room. At these places stainless steel test bodies holders were placed which carried up to ten single stainless steel standard test bodies coated with the defined test microorganisms (*Enterococcus hirae*, DSM 682; ATCC 10536), *Escherichia coli*, DSM 3320; ATCC 10541; Fig. 1). Test bodies were inoculated using a fixed number of test bacteria (1.5×10^9 to 5.0×10^9 per mL) and defined high protein pollution (10 g per mL bovine serum albumin + 10 g per mL yeast extract).

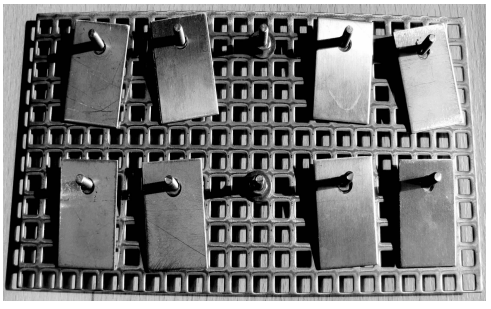

Fig. 1 Stainless steel test body holder with 8 stainless steel test bodies

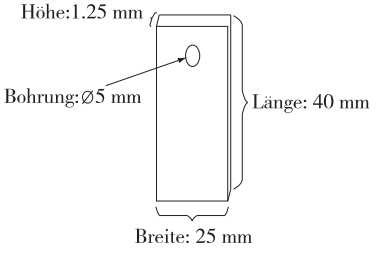

Fig. 2 Inoculation of test bodies with *Enterococcus hirae* and *E. coli*

The inoculated test bodies were disinfected using the biocide Ascarosteril AB consisting of component A (surface-active agent + antiparasitic agent) and component B (peracetic acid of solvent-cage-type) as well as the cold

fog producing apparatus "Automatic DK" provided by Pfalz Technology company(Fig. 3).

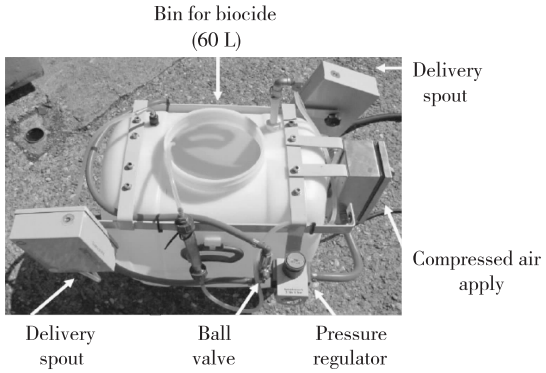

Fig. 3 Cold fog producing apparatus "Automatic DK"

The test bodies were removed from there positions after fumigation and an exposure period of 60 min. To neutralize the biocidal effect test bodies were firstly treated with a neutralization medium. Afterwards, bacteria were re-cultivated and the reduction ratios of the bacterial numbers in relation to disinfection were calculated. The experiments were repeated for four times.

Results and discussion

After optimization four test bodies containing 0.025 mL of each test bacterial suspension and high protein pollution were applied at each of the 12 positions within the room. The drying period for the bacterial suspension was 85 minutes. 8250 mL of a 10% Ascarosteril AB solution was fumigated over a time period of 58 minutes. The results are summarized for *E. hirae* in Fig. 4 and for *E. coli* in Fig. 5.

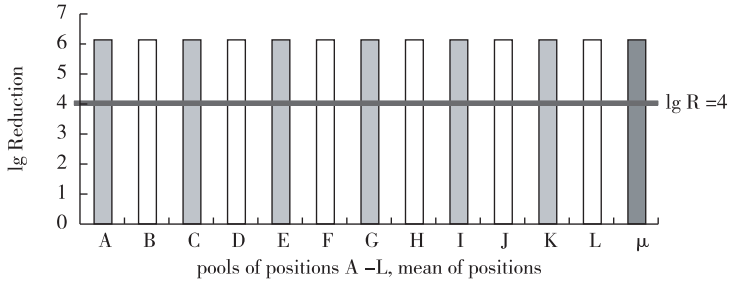

Fig. 4 Results of the pooled samples of the test bodies carrying *E. hirae* and a high protein pollution

In all cases and positions the numbers of both test bacteria were reduced under high protein pollution by up to six log potencies. This means that a disinfection using the described biocide in combination with fumigation was successful.

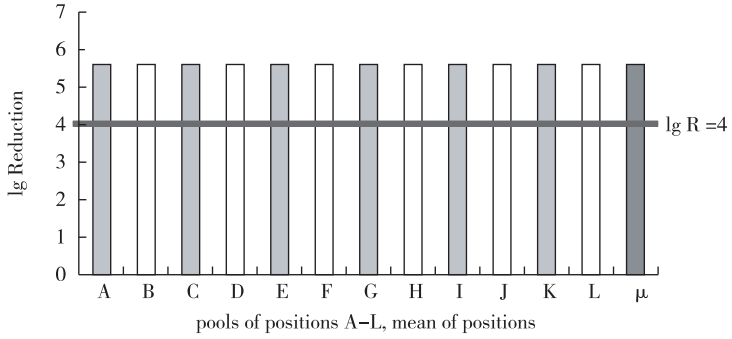

Fig. 5 Results of the pooled samples of the test bodies carrying *E. coli* and a high protein pollution

Conclusions

Our investigations showed that a sufficient disinfection can be achieved by aerosol methods. The success is dependent on the amount and the concentration of the applied disinfectant. Based on our results we found that even 33.56 mL per m of a 10% disinfectant based on peracetic acid are enough to reduce the number of *Enterococcus hirae* and *Escherichia coli* in the tested room by at least four log steps.

In regard to economic aspects our experiments showed that aerosol disinfection is despite the higher investment costs cheaper (up to 30%) than wet disinfection. This is due to the higher working load and therefore, the higher working costs.

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The Effect of Sanitation of Housing Structures on Calf Performance and Health

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Summary: The aim of this study was to determine the efficiency of cleaning and disinfection of pre-weaning calf hutches and the effect on performance and health of calves. Ten individual calf hutches in a dairy operation were included in the two-year experiment. All the hutches were mechanically cleaned after the weaning of calves. Then half of the hutches were exposed to foam washing with alkaline detergent and disinfection with iodine solution. Microbial contamination of hutch surfaces was determined by swabbing the surface area right after the weaning and removal of calves, after mechanical cleaning, after foam washing and after disinfection. In each swab total bacterial count(TBC) was determined, in accordance with the Czech national standards. The health and body weight were monitored in 98 calves. The experimental data were analysed by the StatSoft statistical package. After the removal of calves from the hutches, total bacterial counts on the inner surfaces wall were ranging from $< 1.0 \times 10^1$ to $> 1.5 \times 10^7$ CFU · 10 cm^{-2}. The mechanical cleaning decreased TBC by about 93%. The efficiency of foam washing followed by disinfection of hutch surfaces reached 99%. The results indicate there were no significant effect of disinfection on average daily weight gain and health of calves.

Introduction

Newborn animals are very susceptible to disease [1], as their immune systems are not unprimed [2]. Most diseases of pre-weaned dairy calves are enteric or respiratory ones [3]. Diarrhoea can be a major cause of poor growth and calf mortality in the first month of life in many dairy herds [4]. Respiratory infections are particularly common in calves between 8 and 20 weeks of age [5]. Most pathogens (Rotavirus, Coronavirus, Cryptosporidium, *Escherichia coli*, *Salmonella*, bovine herpes virus 1, bovine respiratory syncytial virus, parainfluenza 3 virus, *Mycoplasma bovis*, *Pasteurella multocida*, *Mannheimia haemolytica*, etc.) become infective through inhalation or fecal-oral contact [5 – 7]. Keeping bacterial populations low in their environment is essential [8]. The major source of infection for calves is the calf hutch environment. Infected calves maintain contamination of these areas [9]. While most hutch environments are power cleaned and allowed to dry between uses, this is likely not enough to destroy pathogens such as Salmonella. Therefore once again a disinfectant applied after cleaning should be considered [8]. Cleaning and disinfection of calf hutches is an integral part of the principles of good husbandry practices and one of basic preventative measures in calf rearing in the period of milk nutrition.

The aim of this study was to determine the efficiency of cleaning and disinfection of calf housing in the pre-weaning period and the effect on performance and health of calves.

Material and methods

The level of cleaning and disinfection of individual calf housing structures was evaluated continually during the two year period (2011 to 2012). Ten individual hutches for pre-weaning calves from birth to about 58 – 60 days of age were included in the experiment, out of these 6 hutches were outdoors and 4 were sheltered. The hutches were made from wood, blue tarpaulin and plastic (white polyethylene). All the hutches were mechanically cleaned after the weaning of calves, using high pressure washers. After the washing, 5 hutches were assigned to an untreated control, whereas the other five (treated) were subjected to foam cleaning with 0.5% solution of alkaline detergent (at a dose of 0.3 litres per $1 m^2$), applied by a foam lance. After 20 – 30 minutes of action, the foam was washed off by pressure washers. After the drying off, all the surfaces were disinfected with 1.0% iodine solution (0.3 litres of iodine solution per $1 m^2$), applied as foam again.

Microbial contamination of hutch surfaces was ascertained by swabbing immediately after the removal of calves, after the mechanical cleaning, after the foam washing and after the disinfection. The successive swabs were always taken from the same place about a surface area 10 cm^2. The swabs were used for the determination of total bacterial count(TBC), i. e. colony forming units (CFU) per 10 cm^2, in accordance with the Czech national standards.

The health status and live weight were monitored in 68 Holstein calves and in 30 Czech Simmental calves. All

calves were fed colostrum from birth to 2 – 4 days of age. From 3 – 5 days to the weaning the calves received a ration composed of milk replacer and calf starter feed at restricted intake. All the calves were provided feed and water twice a day. The bedding in the hutches was covered with 0.5 to 0.7 kg of new straw every two days.

Raw data were analysed by the StatSoft statistical package. The qualitative parameters (health status of calves, total bacterial counts) were evaluated by non-parametric Kruskal-Wallis ANOVA. Average daily weight gain in the pre-weaning calves was analysed by general linear model (GLM), which enabled to take into account the effect of sex of calves.

Results and discussion

The levels of microbial contamination of inner wall surfaces of individual pre-weaning calf hutches are given in Table 1. Performance of pre-weaning calves during the study period, i. e. average daily weight gain, is summarized in Table 2.

Table 1 Microbial contamination of surfaces of individual calf hutches in various phases of sanitation

Phase of sanitation	n	TBC (CFU·10 cm^{-2})			The efficiency of sanitation (%)
		Median	Minimum	Maximum	
After removal of calves	69	$2.40 \times 10^{3\ A,B,C}$	$< 1.0 \times 10^{1}$	$> 1.5 \times 10^{7}$	–
After mechanical cleaning	56	$1.65 \times 10^{2\ A,a}$	$< 1.0 \times 10^{1}$	$> 3.0 \times 10^{4}$	93
After foam washing	27	$6.5 \times 10^{1\ B}$	$< 1.0 \times 10^{1}$	1.00×10^{3}	97
After disinfection	22	$1.9 \times 10^{1\ C,a}$	$< 1.0 \times 10^{1}$	4.60×10^{2}	99

Statistical significance: ^{A,B,C} ($P < 0.01$); ^{a} ($P < 0.05$)

Total bacterial counts on the inner surfaces after the removal of calves ranged from $< 1.0 \times 10^{1}$ to $> 1.5 \times 10^{7}$ CFU per 10 cm^2 (Table 1). Fotheringham [10] showed that numbers of microorganisms on surfaces in animal housing may exceed 10^9/cm^2. Mechanical cleaning resulted in 93% reduction in total microbial counts. According to Fotheringham [1], mechanical cleaning alone can remove over 90% of bacteria from the surface. Cleaning should be reduced, depending on the kind of material, total bacterial count by at least 3 log orders [11]. Total bacterial count after the removal of calves was proven statistically significantly higher ($P < 0.01$) than the values determined after the mechanical cleaning, after the foam washing or after disinfection. The foam washing reduced total microbial counts on the inner surfaces to 6.5×10^{1} CFU · 10 cm^{-2}. Total bacterial counts decreased after subsequent disinfection to 1.9×10^{1} CFU · 10 cm^{-2} surface (Table 1). The efficiency of foam washing followed by disinfection of surfaces reached 99%. The aim of disinfection is to lower the microbial load on surfaces to a level which causes neither the spread of pathogens (less than 10^4 CFU/cm^2) nor a reduction in animal productivity [10]. Efficient cleaning/disinfection can remove up to 99% of bacteria present [12]. Full devitalization of microorganisms in a breeding environment cannot be achieved by conventional sanitation methods [13]. Disinfection should reduce total bacterial count by 3 log orders. This means that under farm conditions generally 10^3 CFU of bacteria remain per cm^2 of surface, mostly sporeformers [11]. After the disinfection the maximum total bacterial count on the surface was 4.6×10^{2} CFU · 10 cm^{-2}, therefore the disinfectant can be regarded as effective ($\leqslant 5 \times 10^{4}$ CFU · 10 cm^2), according to State Veterinary Administration of the Czech Republic [14].

Table 2 Average daily weight gain of pre-weaning calves by sex and sex and level of housing hygiene

Method of sanitation	Sex of calves	n	Mean (kg)	Standard deviation (kg)
Mechanical cleaning	male	22	0.607	0.134
Mechanical cleaning + foam cleaning + disinfection	male	25	0.636	0.146
Mechanical cleaning	female	34	0.602	0.113
Mechanical cleaning + foam cleaning + disinfection	female	17	0.511	0.134

Average daily weight gain of pre-weaning calves ranged from 0.511 to 0.636 kg. According to Kertze et al. [15] the average daily gain of heifer-calves fluctuated from 0.680 to 0.770 kg during the first two months of life. Diaz et al. [16] showed that average daily gain of bull-calves can move from 0.560 kg to 1.100 kg from birth to about 8 weeks. Our results of average daily gains (Table 2) were somewhat higher than 0.408 kg found by VandeHaar [17] in calves fed commercial milk replacer and calf starter at restricted intake.

Bull-calves housed in disinfected individual housing, achieved higher average daily gain (about 0.029 kg), while average daily gain of heifer-calves housed in disinfected housing was lower by 0.091 kg than heifer-

calves housed only in mechanically treated of individual housing(Table 2). There was, however, no significant difference between average daily gain of bull calves, nor between average daily gain of heifer calves housed in disinfected and non-disinfected (only mechanically cleaned) individual hutches.

In the disinfected hutches the same number of calves (21 calves) manifested diseases(diarrhoea, diseases of respiratory tract) as in the hutches, which were mechanically cleaned only(22 calves). There were no significant differences between the occurrence of sick and healthy calves housed in disinfected hutches (experimental group; 21 healthy calves versus 21 calves with diarrhoea or with diseases of respiratory tract) or non-disinfected(control group; 22 healthy calves versus 34 sick calves) individual housing of dairy calves.

Conclusions

The results indicate that no significant effect of disinfection on average daily weight gain and health of calves demonstrated. Cleaning and disinfection of calf housing is an integral part of the good husbandry practices as one of the basic preventative measures in calf rearing and one of the pillars of biosecurity. The choice of sanitation method of individual pre-weaning calf housing structures depends directly on the analysis of epidemiological status on the farm. For herds with good epidemiological status a system of dry cleaning with subsequent washing can be regarded as satisfactory. On the other hand, in herds with unfavourable epidemiological situation and a large concentration of animals, low-pressure washing with subsequent disinfection should be used before allocation of calves, in addition to mechanical cleaning.

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Preventive Veterinary Medicine and Herd/Flock Health Management

Molecular Epidemiological Studies on Bovine Theileriosis in Upper Egypt

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Summary: The results of the current study concluded that the conventional method of diagnosis (blood films) of theileriosis was still recommended for day to day examination of clinically infected cases, especially in acute stage which gave (28.57%) while the molecular assay (Tams-1 target based PCR test) gave 46.19%. The infection rate were higher in ELWady EL-Geded governorate followed by Assiut and EL-fayoum governorates. Frisian breed of cattle were more sensitive and susceptible than native breeds. The animals below one year old were more susceptible to infection, if compared with the older animals. The female animals were more susceptible to the disease than males. The high level of the infection rate with tropical theileriosis is more common during hot-months than non-hot months.
Key words: cattle, tropical theileriosis, Upper Egypt, conventional diagnosis, Tams-1 PCR

Introduction

Bovine theileriosis in Egypt is a tick-borne disease known as Egyptian fever since 1947, caused by protozoan parasite known as *Theileria annulata*. The disease is one of the most destructive obstacles to the livestock production in Egypt [6]. It transmitted by ticks of genus Hyalomma. Early and accurate diagnosis plays a crucial role for thileriosis control. In early phase, it can be easily diagnosed by Giemsa stained thin blood film. Also, by direct visualization of the schizont which infected lymphocytes and monocytes in Giemsa-stained biopsy smears. The application of genotypic assays for the diagnosis of bovine thieleriosis has shown recent advances [15]. Polymerase chain reaction (PCR) offers important advantage such as the greater sensitivity and specificity over conventional techniques in detecting both piroplasm-infected and carrier animals. This has been verified in a number of studies performed on a wide range of animals [2, 4, 15, 28].

Material and methods

Materials

Animals: A total number of 210 cattle belong to different localities in EL-Fayoum, EL-Minia, Assuit, Sohage and EL-Wady EL-Gaded governorates were subjected to this study (Table 1). All animals showed acute or chronic forms of tropical theileriosis with different degrees of tick infestation. Two types of blood samples were collected from each animal, one collected directly from the ear vein and used for preparation of blood films, another from jugular vein collected on EDTA vacationer tubes used for DNA extraction then stored at −20 ℃ till use[12, 13].

Methods

① Clinical Examination: All animals in this study were subjected to clinical examination according to [25].

② Conventional diagnosis: Thin blood films after fixation stained with Giemsa stain diluted at 8% and examined on Olympus microscope by using Oil immersion lens at ×1000 magnification [14, 15].

③ Molecular diagnosis: For genitical confirmation of *T. annulata* infection, the *T. annulata*-specific target (Tams-1) sequence was amplified using polymerase chain reaction (PCR). Primer Tams1 F (5\ ATG CTG CAA ATG AGG AT) and Tspms1 R (5\GGA CTG ATG AGA AGA CGA TGA G), Amplifying a (785 bp) fragment of the *Theileria annulata* 30 KDa major merozoite surface antigen gene, Tams1, were used [9, 23, 24].

DNA Extraction: DNA extraction from whole blood samples was carried out according to commercial kits (manufacturer's instructions of QIA amp blood kit, Qiagen, Ltd, UK).

Cycling conditions: PCR was performed by incubating the samples at different three temperatures corresponding to three steps (Denaturation, Annulling, Extension). 94℃ for 5 min., followed by 37 cycles consisting of 1 min at 94℃, 1 min at 55℃, 2 min at 72℃ and final extension step at 72℃ for 10 min. Longer then the samples were stored at 4℃ until use it in the next step. The cycling condition carried out in thermocycler TECHNE TC-312.

Gel Electrophoresis: 1.8% agarose gel (GX 040. 90, Gen AGarose, L. E., Standard DNA /RNA agarose, Molecular Biology Grade, Inno Train Diagnostic, D-61476, Kronberg/Taunus) Containing Ethidium bromide as 1 μl/ml electrophoresis buffer. The image of the PCR

products containing the DNA sequence of 785 bp were amplified using DOC-It® LS, Image acquisition software (UVP, INC, UK).

Results and discussion

The clinical examination revealed various degrees of tick infestation. The clinical signs includes fever (41 – 42℃), enlargement of superficial lymph nodes, various degrees of respiratory affections were also recorded and Ocular lesions like corneal opacity. Similar clinical signs were recorded in previous studies in both cattle and buffaloes [1, 7, 5, 17] all reported the same clinical signs in cattle. The rate of conventionally confirmed infection among examined animal revealed that the percentages of infection were 28.57%. On the other hand, the infection rate according Tams-1 target based PCR was 46.19%. These finding was supported by previous finding of [22, 27] in Germany and Sudan. [1, 7] in Upper Egypt. These results concluded that PCR test was more sensitive than other diagnostic assays. This come in agreement with [7] in Upper Also [15] in Mauritania, [3, 4] in Turkey, [10, 16] in Iran and Pakistan, respectively.

Relation between the epidemiological factors and rate of molecular-confirmed infection among clinically suspected cases

The locality: The infection rates were higher in El-wady El-geded, Assiut and El-fayoum, governorates (27.62%, 10.95% &7.62%). This may be attributed to the high temperature of the atmosphere in these areas and the variable activity of tick vectors and its eradication programs in these different localities this finding come in agreement with [1, 7] in Upper Egypt.

Breed susceptibility: From the previous results it cleared that Frisian cattle were the most susceptible animals to *T. annulata* infection. Its infection rate percentage was 37.62% if compared with the native breed of cattle which recorded less susceptibility 8.57%. These come in agreement with [7, 20] in Egypt; [11, 27] in Sudan; [21] in Germany all of them reported that local had tolerances to infection while exotic and cross breeds. The lowering of infection rate in cases of native cattle may be due to the balance between the infection and the animal's immune status, which occur due to recurrent infections and treatment specially in endemic areas. The animal considered as carrier and played an important role in transmission of infection to other new healthy introduced animals.

Sex susceptibility: Females were more susceptible than males, the percentage rate of infection in females were 25.24% while in male was 20.95%. This result was agreed with [19] in the Cappadocia Region of Turkey who reported higher infection rate in females cattle (87.6%) than that in males (12.4%).

Age susceptibility: Age plays an important role in the animal susceptibility to infection, from the obtained results animals less than one year old have the higher infection rates (25.24%) Followed by animals that less than three and below five years old in both the infection rates were (8.09%). Finally animals above five years old showed infection rate (4.76%). These results agree with [26] in Sudan. [17] In El-Mansoura and [7] in Upper Egypt. This may be attributed to the cumulative antibodies against the causative agent of the disease in case of oldest animals due to previous infection, while in young animals the antibody titer is usually low so these animals have low resistance and so not fully protected against the disease specially if those animals introduced for the first time to an endemic area.

Seasonal variation: Seasonal variation also play an important role and had a direct effect on the incidence of this disease in both animal species out from the obtained results in this study the infection with tropical theileriosis is more common during hot months with infection rates (28.57%) followed by non-hot months (17.62%) this may be due to increase the activity of the biological tick vector during these times from the year. These results in agreement with [1, 7] in Upper Egypt. On the other hand these results not in agreement with the results that obtained by [17] in El-Mansoura, who recorded that infection rates was more common during autumn months then summer, spring months and recorded the lowest rate during winter months.

Conclusion

This current study concluded that the conventional method of diagnosis (blood films) was still recommended for day to day examination of clinically infected cases, especially in acute stage, while the molecular assay is recommended for the epidemiological studies. The infection rate higher in ELWady EL-Geded governorate followed by Assiut and EL-fayoum governorates. Frisian breed of cattle were more sensitive and susceptible than native breeds. The animals below one year old were more susceptible to infection, if compared with the older animals. The female animals were more susceptible to the disease than males. The high level of the infection rate with tropical theileriosis is more common during hot-months than non-hot months.

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The Use of Vaccines and Medicine Like Antibiotica and Other Drugs in the PR China Experiences of a Veterinarian During Visits in Pig Farms in Chinese Provinces

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Summary: In the PR China there are diseases under the pigs. By vaccination programs the diseases are reduced. The Ministry of Agriculture, bureau of veterinary, Beijing has installed in the provinces animal control centers. The stuffs have to visit the farms for taking urine-and blood samples for reduce the use of forbidden drugs, antibiotica use and for control the pig vaccinations in the farms.

Introduction

In the year 2006 by the Ministry of Agriculture (MOA) the PR China [4] is installed a disease control system for the main epidemic diseases and since 2009 a vet system for regulations of veterinary drugs about the use of antibiotic and drugs. In the provinces are animal control institutions with vet stations. The pig farmers and the agricultural vets protect the herd health by urin and blood probes, laboratory diagnostic and vaccination tools [1] during the pig live.

Material and methods

In the PR China there are private farms with a few sows and with more than 1000 sows. In the years 2008 – 2012 in five provinces during the farm visits in breeding farms we talked with the farmers about the health status, the vaccination programes and the use of drugs for the pigs during the production. The antibiotica, vaccines and other medice are transmitted by distributors from the pharma industry directly to the pig farms. There the medicin is used by the agrar-vets, to treat the pigs against specially diseases. The agrar vet stations with 140738 stuffs in the year 2012 in the provinces are responsible for the vaccines and the disease reportings to the MOA in Beijing. During the live the fatteners were tested by urine probes and blood samples some days before slaughtering by the staffs of the animal health inspection service. At the slaughterhouses the fatteners were tested for antibiotica and drugs. The results were send to the health inspection offices and to the MOA.

Results

Since several years the farmer have problems with new diseases as PRRS- and Circo Virus infections under the pigs [3]. With the vaccination (Table 1) against different diseases, we could find, that the losses during weaning, in the flats and the fatteners, were reduced. In China for many diseases home maid vaccines with special phyloms for reducing the losses are produced. After the use of the Boehringer Ingelheim (BI) Ingelvac MLV PRRS live vaccine during our visits in the farms we found a high health status under the pigs with a good productivity. With this commercial BI vaccine the performance was higher than with home maid PRRS vaccine strains.

In the PR China Agricultural Department regulation 193, to protect peoples food safety and peoples health are written:

1. order to the animal medicine control
2. a list of forbidden medicine to produce
3. a list of forbidden medicine to put in the feed or the water.

The list of forbidden drugs contains 21 chemicals as clenbuterol, hormons (diaethyl-stilbestrol, testosterone etc.), antibiotica, sulfonamids, plans protect drugs, anaestetica etc.. The results of the laboratory tests from each farm are send directly to the MOA. During my farm visits I could not see the test results.

Discussion

In closed herds with sows and fatteners there are more problems than with a two or three side production systems. Many diseases as PMWS and PCVD can be controlled by the licensed commercial vaccines tested and produced by international pharmaceutical companies. Some authors have focused, that "Porcine High Fever Disease" (PHFD) in China is caused by a highly pathogenic PRRS strain [2]. During the farm visits we have found, that the BI PRRS vaccine can be used successful against these highly pathogenic PRRS strains in China. In US test farms with this high pathogenic PRRSV strain there was no statistical difference between the effect of the infection with strains of high virulence and groups vaccinated with a autogenous killed vaccine product. The agrar-vets and other staffs in the provinces control the

Table 1 Vaccine program for pig farms in the PR China

Group	Week/Age	Vaccine times	KV/MLV	Company
piglet	2 – 3 week	M. hyo 2x	KV	BI
piglet/sow/boar	1 – 3 day i. nasal. 8 – 10 week i. nasal	M. Auj 2x 3 – 4x	MLV	BI
piglet/sow/boar	7 – 14 day mass vacc. /year	PRRS 1x 3 – 4x	MLV	BI
piglet/pigs	> 2 weeks	PCV2 1x	KV	BI
gilts/sow	a. insemination p. insemination	PPV 2x 1x	KV	IV
sow	3 – 6 week before farrowing.	AR 4 1x	KV	BI
piglet/sow	2 – 3 week 4 week before farrowing	HP 1 1x 1x	KV	BI
piglet	1 week before weaning	Ileitis 1x	MLV	BI
piglet/sow/boar	wint: 45 + 90 d sum: 70 + 100 d per year	FMD 2x 3 – 4x	KV	home
piglet/sow/boar	20 + 60 days weaning	CSF 2x 3 – 4x	MLV	home
piglet/sow/boar	3 – 4 week 5 week before farrowing	SS 1x 1x	KV	home
sow	3 week before farrowing	PED 2x	KV	home
gilt/sow	march/april year	JE 2x	KV	home
piglet/sow	45 days after farrowing	Erysip 2x 2x	KV	home
piglet/sow	45 days 2 week after farrowing	Past. 1x 1x	KV	home

Abbreviations: BI: Boehringer Ingelheim, IV: Intervet

fatteners for drugs, to produce save pig food.

Abstract

The pig production in big herds is growing the last ten years to 50% of the world production. For reducing epidemic diseases as FMD, CSF, M. Auj. or Japanese Encephalitis and other diseases as PRRSV, RA, PPV etc. by the Ministery of Agriculture (MOA) in the PR China are installed in the provinces bureaus with veterinaries and laboratories for testing the diseases, the vaccinations, the residuals of antibiotica and the unload use of forbidden drugs in the pigs. These standards improve the biosecurity and a save pork production, to protect people for food safety and for people health.

As a member of the FDA. WTO and the OIE organisations the chinese government has installed rules for biosecurity and for a safe pork production to producing animal food with international standards.

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Evaluation of Ultrasonography as a Diagnostic Tool for Hepatic Cystic Echinococcosis in Sheep

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Summary: This study aimed to gain information about ultrasonographic findings in sheep with hepatic cystic echinococcosis as well as to evaluate the use of ultrasonography for diagnosis of such infection, furthermore, clinical, hematological and biochemical variations were also investigated. This study was conducted on 22 sheep of both sexes, which classified into two groups according to their liver health group (healthy liver = 13 and cystic liver = 9). Ten sheep were slaughtered and a comparison between ultrasound and post-mortem results was done. Biochemically, serum concentrations of γ-glutamyl transferase, aspartate aminotransferase, total bilirubin, and globulins were significantly increased ($P < 0.01$), while albumin was lowered ($P < 0.01$) in sheep with cystic liver. Ultrasonographic findings of diseased sheep livers revealed presence of rounded, anechoic and unilocular hydatid cysts with ellipse circumference ranged from 6 to 10 cm. The borders of cysts were mostly well defined. The interior of cysts contained echogenic particulate materials, septations, or fine echoes. At the 10th intercostal space, all of ventral margin, size, thickness, and angle of livers were higher in sheep with hepatic cysts than healthy ones ($P < 0.01$), while the diameter of portal vein was lesser in sheep with liver cysts than control ones ($P < 0.01$). Furthermore in comparison with healthy liver group, at 9th intercostal space, the circumference of gall bladder was lowered in sheep with hepatic cysts ($P < 0.01$). The sensitivity, specificity, positive and negative predictive values of ultrasonography for diagnosis of hepatic hydatid cysts were 80% and 100%, 100%, and 83%, respectively. In conclusion, as an objective non-invasive practical tool, ultrasonography alone or in combination of some biochemical parameters reflecting liver function could be helpful for diagnosis of hepatic hydatid cysts in sheep.

Introduction

Cystic echinococcosis (CE) is a zoonotic parasitic infection of many mammalian species caused by the larvae of *Echinococcus granulosus*, which is found in the small intestines of dogs and other carnivores [1]. Sheep, cattle, and camel are considered as intermediate hosts [2]. Furthermore, Liver and lung cysts of *E. granulosus* are a worldwide parasitic disease [3] and is endemic in countries where sheep grazing is carried out with the help of dogs [4]. Sheep as intermediate host are infected by ingestion of parasite eggs, which reach the liver via the portal system to form hydatid cysts [5]. Liver is involved in up to 75% cases but no part of the body is spared [6]. Cystic liver disease has economic impact in countries where livestock industry is an important segment of the agricultural sector and when livestock production is based mainly on extensive grazing system [7]. Significant losses are of special significance in countries with low economic outputs where sheep production is of particular importance [8] including losses of meat and milk production and fleece values from infected sheep may also occur [9]. There are no reliable methods for the routine diagnosis of liver cysts in living animals, but in rare cases cysts have been identified by ultrasonography alone or in conjunction with serum antibody detection [10]. This study was planned to gain information about ultrasonographic findings of sheep liver with hydatid cysts, furthermore, to assess the use of ultrasonography for diagnosis of hepatic hydatid cysts.

Material and methods

Animals, history and physical examination

In this study, a total number of twenty two Baladi sheep (aged 3 – 6 years) of both sexes were admitted because of weight loss, diarrhoea, and pregnancy diagnosis at the Veterinary Teaching Hospital, Assiut University, Egypt. Case history of infected sheep revealed grazing on pastures nearby stray dogs' districts or with the help of dogs. This information was obtained from the owners after a questionnaire was prepared for this study. Based on clear hepatic ultrasonographic findings, all animals were classified into two groups as with hepatic cysts ($n = 9$) and without liver cysts (healthy liver, $n = 13$). All animals were subjected to physical examination as described previously [11]. This included their general behaviour and condition; auscultation of the heart, lungs, rumen and intestine; measurement of heart rate, respiratory rate and rectal temperature. Animals with other disease conditions were excluded from the study.

Hematological and biochemical analyses

Two blood samples were collected by puncture of the jugular vein, one with heparin and the other without anticoagulant. Blood gas analysis and a complete blood count including hematocrit, hemoglobin, erythrocyte count, and total leucocyte count were carried out on the first sample [12]. After centrifugation of the second blood sample, serum samples were collected and then frozen at −20℃ for one week, later on spectrophotometric analysis of biochemical parameters including total proteins, albumin, blood urea nitrogen, creatinine, total bilirubin, aspartate aminotransferase (AST) and γ-glutamyl transpeptidase (GGT) was carried out.

Ultrasonographic examination

Ultrasound examination was carried out while the animals were standing using real time B-mode scanner with 3.5, 5.0, and 8 linear and sector transducers (Veterinary Ultrasound Scanner, Esoate Europe B. V., the Netherlands). In preparation of ultrasonography, the right thorax and abdomen were clipped, shaved and a coupling gel was applied. Ultrasonographic examination of liver was performed on the right side of the abdomen in the twelfth through seventh intercostal spaces. In each intercostal space, the dimensions of the liver, and if visible, the location and diameter of the caudal vena cava and portal vein were determined, furthermore, the angle of the liver, and location and circumference of the gall bladder were also determined [13]. In addition number, size and location of the cysts were noted.

Statistical analysis

Data are presented as Mean ± SE and the analysis was conducted using SPSS program, version 16.0. Hematological, biochemical, and ultrasonographic findings were compared using Student's t test. A contingency 2 × 2 table was created to compute the sensitivity, specificity, positive and negative predictive values using ultrasonography. The formulas described later were used to calculate the sensitivity, specificity, positive and negative predictive values [14]: Sensitivity = 100 × [true positive/true positive + false negative], Specificity = 100 × [true negative/true negative + false positive], Positive predictive value = 100 × [true positive/test positive], and Negative predictive value = 100 × [true negative/test negative].

Results

Clinical, hematological, and biochemical findings

Case history of diseased sheep revealed grazing on pastures nearby stray dogs' districts or with the help of dogs. This information was obtained from the owners after a questionnaire was prepared for this study. Clinically, sheep with cystic liver revealed in appetence with frequent diarrhoea and constipation. Two cases had roughness of wool and shaggy appearance. Table 1 shows the haematological and biochemical findings in healthy sheep and diseased ones.

Table 1 Hematological and biochemical findings of sheep with two liver health groups (n = 22)

Parameters		Liver health group	
		Cystic liver (n = 9)	Healthy liver (n = 13)
Hematocrit (%)		34 ± 1.0	32 ± 0.6
Hemoglobin (g/L)		98 ± 3	100 ± 2
Erythrocytes (T/L)		9.2 ± 0.18	9.6 ± 0.24
Leukocyte count (G/L)		6.4 ± 0.17	5.9 ± 0.15
γ-Glutamyl transferase (U/L)		46 ± 3**	33 ± 2
Aspartate aminotransferase (U/L)		104 ± 4***	36 ± 1
Total bilirubin (μmol/L)		8.5 ± 0.55**	4.2 ± 0.40
Total proteins (g/L)		69 ± 1	70 ± 0.8
Albumin (g/L)		22 ± 0.5**	28 ± 0.44
Globulins (g/L)		47 ± 1.3**	42 ± 0.8
Albumin/globumin ratio		0.5 ± 0.06**	0.7 ± 0.02
Blood urea nitrogen (mmol/L)		3.9 ± 0.16	4.2 ± 0.15
Creatinine (μmol/L)		111 ± 2.0	114 ± 1.5
Venous blood gas and acid-base indices	pH	7.37 ± 0.02	7.36 ± 0.01
	HCO_3 (mmol/L)	22 ± 0.3	23 ± 0.2
	tCO_2 (mmol/L)	24 ± 0.5	25 ± 0.4
	pO_2 (mmHg)	34 ± 0.3	35 ± 0.3
	pCO_2 (mmHg)	42 ± 0.6	44 ± 0.5
	BE (mmol/L)	0.42 ± 0.04	0.40 ± 0.07

Data presented as Mean ± SE, ** P < 0.01; *** P < 0.001.

Ultrasonographic findings

Livers of all animals were examined from the twelfth to the seventh intercostal spaces. In one healthy sheep, the 12th intercostal space was narrow; therefore ultrasonographic examination was not possible in this place. At the 10th intercostal space, all of ventral margin

(20 ± 0.33 vs 18 ± 0.24), size (11.6 ± 0.22 vs 9.5 ± 0.26), thickness (9.2 ± 0.09 vs 8.3 ± 0.29), and angle (38 ± 1 vs 35 ± 0.9) of liver were higher in sheep with liver cysts than healthy ones ($P < 0.05$). Out of 9 cases with hepatic cysts, 3 cases showed cysts at the right lobe of liver near the portal vein with increased echogenicity of its wall and narrowing of its diameter (Fig. 1), and two cases exhibited cysts near the angle of liver at 10th intercostal space (Fig. 2). The parenchymal pattern of liver in control sheep consisted of numerous weak echoes homogenously distributed over the entire liver (Fig. 3), whereas in diseased sheep the liver showed heterogeneous hyperechogenic parenchyma (Fig. 1 and 2). In general, liver cysts were rounded, anechoic, unilocular structure with typically hypoechogenic contents (Fig. 2). Ellipse circumference of hepatic cysts ranged from 6.7 cm to 10.4 cm. The ultrasonographic appearance of liver cysts was homogenous in five animals and heterogeneous in rest of animals. The interior of some cysts contained echogenic septations (Fig. 1), particulate materials (Fig. 4), or fine echoes. The borders of cysts were either ill defined (Fig. 1) or well defined (Fig. 2). The caudal vena cava was located dorsal and medial to the portal vein. It was usually triangular on cross-sectional view (Fig. 1, 3), whereas the portal vein was round or slightly oval (Fig. 1, 2, 3). In both health groups, the diameter (1.4 ± 0.06 vs 1.7 ± 0.05) of caudal vena cava increased cranially, whereas the portal vein diameters (1.8 ± 0.09 vs 1.2 ± 0.05) decreased cranially ($P < 0.05$). The dorsal margin, depth, and diameter of caudal vena cava showed insignificant difference between the two liver health groups ($P > 0.05$), whereas at 10th intercostal space, the diameter of portal vein was lesser in sheep with hepatic cysts than control ones (1.2 ± 0.06 vs 1.5 ± 0.03) ($P < 0.01$). In the two liver health groups, gall bladder was visualized in all sheep. Generally, gall bladder was visible in ninth or tenth or both intercostal spaces. Ultrasonographically, the gall bladder was recognized as a fluid-filled vesicle, which appeared oval or pear-shaped dark area with bright margin (Fig. 3). At 10th and 9th intercostal spaces, dorsal margins of gall bladder were greater in sheep with hepatic cysts than healthy one (16 ± 0.58 vs 12 ± 0.13) (27 ± 0.35 vs 23 ± 0.28) ($P < 0.01$), respectively, whereas at 9th intercostal space the circumference of gall bladder was lesser in diseased group than control ones (11 ± 1.1 vs 16 ± 0.3) ($P < 0.01$).

Post-mortem findings

In order to evaluate the reliability of ultrasonography for detection of hepatic cysts, ten animals were slaughtered after ultrasonographic examination as well as owners' agreement. Necropsy examination of two cases

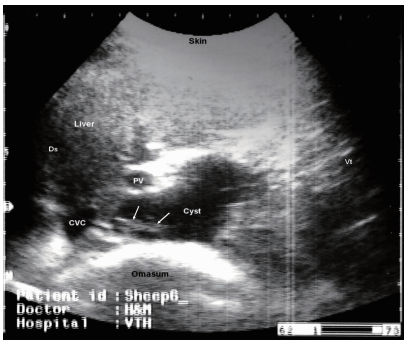

Fig. 1 Ultrasonogram of liver of a 5-year-old sheep viewed from 9th intercostal space with a 5-MHz sector transducer showing a periportal cyst with ill defined borders and anechoic content containing echogenic septa (white arrows) (Note narrowing with hyperechogenicity of portal vein) CVC: Caudal vena cava, PV: Portal vein, Ds: Dorsal, Vt: Ventral.

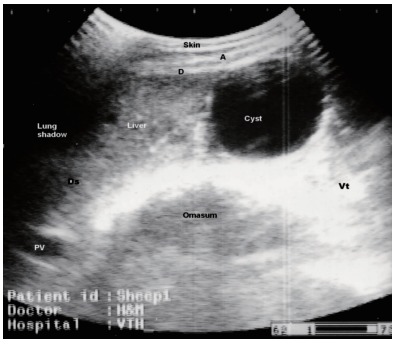

Fig. 2 Ultrasonogram of liver of a 4-year-old sheep viewed from 10th intercostal space with a 5.0-MHz sector transducer showing anechoic content of hepatic cyst near the liver angle with increased echogenicity of liver parenchyma. A: Abdominal wall, D: Diaphragmatic surface, PV: Portal vein, Ds: Dorsal, Vt: Ventral.

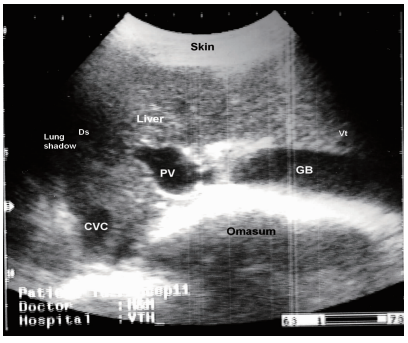

Fig. 3 Ultrasonogram of liver of a 5-year-old sheep viewed from 9th intercostal space with a 5-MHz sector transducer showing grayish echogenicity of parenchyma in a healthy liver and non-compressed portal vein (compare with Fig. 2). CVC: Caudal vena cava, PV: Portal vein, GB: Gall bladder, Ds: Dorsal, Vt: Ventral.

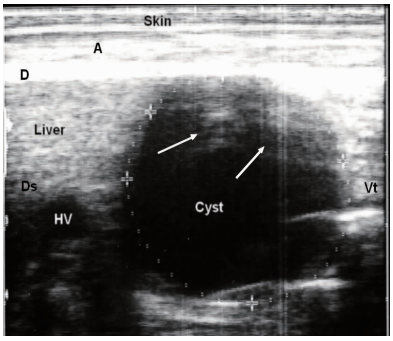

Fig. 4 Ultrasonogram of liver of a 4-year-old sheep viewed from 9th intercostal space with a 8-MHz linear transducer showing a cyst (ellipse circumference = 10.05 cm) with echogenic particulate contents (white arrows) A: Abdominal wall, D: Diaphragmatic surface, HV: Hepatic vein, Ds: Dorsal, Vt: Ventral.

showed that all cysts were located in the right and left lobes of the liver. These cysts were found dead as they were caseous. In three cases, cysts were observed near the portal area. Post-mortem examination of these cysts revealed presence of protoscolecies and clear hydatid fluid indicating viability. Post-mortem examination of the rest of slaughtered sheep (5 cases) revealed no gross hepatic lesions. One positive sheep on post-mortem examination was falsely identified as negative on ultrasonographic examination. In comparison with post-mortem findings, the sensitivity, specificity, positive and negative predictive values of ultrasonography as a diagnostic toll for hepatic hydatid cysts were 80% and 100%, 100%, and 83%, respectively.

Discussion

Although the importance of sheep in the cycle of CE has been recognized, diagnostic methods that allow in vivo identification of parasitized sheep have been poorly evaluated. Serologic tests for the diagnosis of hydatid cysts in sheep have proven unreliable [15] and of limited use [16] as these tests does not distinguish between current and previous infections as well as cross-reactivity between *Echinococcus* and *Taenia* species may occur [16]. Such information is currently obtained at postmortem examination and only at abattoir during veterinary meat inspections. Thus, the only available data are incomplete and only refer to institutional slaughtering [17]. Additional diagnostic techniques are often helpful for evaluation of liver function through estimation of liver enzymes, total bilirubin, total proteins, and albumin [18]. In diseased sheep, the significant increased activities of AST and GGT could be attributed to leakage of these enzymes from hepatocytes as a result of pressure damage caused by hydatid cysts on the liver tissue, whereas increased serum levels of total bilirubin, decreased albumin and lowered albumin globulin ratios might be due to impaired liver function. Previously, Barnes et al. [19] stated that hydatid cysts grow progressively and increase in size and weight, producing pressure atrophy of the parasitized organ and functional alterations.

In ruminants, Hepatic function tests are generally not specific and unable to distinguish between liver diseases [20]. In contrast, ultrasonography may be a quick, non-invasive and well tolerated technique for diagnosis of hepatic cysts in the field. In the present study, in both healthy and diseased sheep, livers were examined ultrasonography from the twelfth to the seventh intercostal spaces except in one healthy sheep, the 12^{th} intercostal space was narrow; therefore ultrasonographic examination was not possible in this place. In sheep with hepatic cysts, increased echogenicity of liver may be due to the changes of the liver tissue nature which may increase the attenuation of the ultrasound beams [21]. Although the significant increase of ventral margin of liver in diseased sheep at the 10^{th} intercostal space, but it remains within the reference range [22].

At the 10^{th} ICS, the significant increase of liver size, thickness and angle in sheep with hepatic cysts might be due to growing of hydatid cyst [19]. Braun and Hausammann [22] concluded that increased liver size in sheep could be suspected when the liver thickness in 1 ICS is >8.5 cm. Generally, the contents of hepatic cysts were anechoic, although the interior of some cysts contained either echogenic particulate materials, which may be corresponding to hydatidic gallstones fine echoes, which may represent the hydatid sand or septations, which may give a daughter cyst. These findings were coincided with Taylor et al. [23].

In the current study, significant decrease of portal vein diameter at 10^{th} ICS could be attributed to the compression caused by hepatic cysts as out of 9 cases with hepatic cysts, 3 cases showed cysts near the portal vein (Fig. 2). In diseased sheep, increased dorsal margin of gall bladder at both 10 and 9^{th} ICS might be due to displacement of gall bladder and the pressure caused by liver cysts. In this work at 9^{th} ICS, although the circumference of gall bladder was significantly decreased in sheep with liver cyst, it is difficult to decide the size is lowered; because the circumference of gall bladder changes daily as described before [22]. Ultrasonography could be used in the diagnosis of hepatic CE because it allows for identification not only the affected organ but also the topographic relationship of the cysts.

In this study, the presence of one sheep as a false negative caused lowering of sensitivity of ultrasound, this could be attributed to the cysts in this sheep were located in the left lobe of the liver, an area not accessible to

ultrasound detection [9]. Results in the present study showed higher sensitivity of ultrasound for diagnosis of CE than previous results (sensitivity = 57.36%) obtained by Sage et al. [24]. Such difference may be due to variations in the number of animals which are false negatives. In the current study, furthermore, as a result of owners' disagreements for slaughtering the rest of infected animals (4 sheep) because of economic and zootechnic reasons; they were treated with oral oxfendazole (Synthatec, 22.5% solution) at 30 mg/kg/day [25]. Unfortunately, these animals were discharged from the hospital, and the authors did not have the opportunity to follow them up for evaluation of treatment.

Conclusion

On the basis of the data obtained in this study, the sensitivity, specificity, positive and negative predictive values of ultrasonography for diagnosis of hepatic hydatid cysts were 80% and 100%, 100%, and 83%, respectively. These signify that using of ultrasonography is a sensitive method for diagnosis of hepatic CE. As an objective non-invasive practical tool, ultrasonography alone or in combination of some biochemical parameters reflecting liver function could be helpful for diagnosis of hepatic cystic echinococcosis in sheep.

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Quantification of the Severity of an Outbreak of Animal Infectious Diseases

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Summary: In this report, a method for measuring the severity of an outbreak of human infectious diseases was firstly reviewed. This method was then used as a premise for the method reported here to measure the severity of an outbreak of animal infectious diseases. It involves scoring the direct economic loss due to the outbreak and outbreak control, transmission of the infection, the public significance of the outbreak, and novelty of the outbreak. Then, it classifies outbreaks of animal infectious diseases into Grade Ⅰ - Ⅳ (Grade Ⅰ represents mild outbreaks, while Grade Ⅳ represents extremely severe outbreaks).
Key words: quantification; severity; outbreak; infectious disease; infection control

Usually, according to the present legislation of many countries, decisions on taking costly emergent measures to control an outbreak of human or animal infectious diseases required the diagnosis of the disease or identification of the pathogen. However, precious time for control of infectious disease outbreak at its early stage could be lost, since the diagnosis or the pathogen identification may be time-consuming or even incorrect, especially for some emerging infectious diseases with pathogens unknown previously. Using severe acute respiratory syndrome (SARS) as an example [1,2], the first human case of SARS occurred in Nov., 2002 in Guangdong, China. By 02/09/2003, 305 cases of SARS with 5 fatalities had been reported there, and it was found that the disease likely could transmit among people without close contact. However, emergent measures, such as isolation of patients and movement restriction, had not been implemented, until the pathogen was correctly identified in March, 2003. Consequently, the outbreak finally spread to approximately 30 countries with more than 8000 probable cases and 800 fatalities (www.who.int/csr/sars/en/). The outbreak of Nipah virus in Malaysia in 1998 is another clear example [3,4]. It began in September, 1998 in a village Ampang in the Kinta district, where several cases of fatal febrile encephalitis in humans followed respiratory and encephalitic diseases in pigs. By 03/06/1999, human encephalitis cases with >40% fatality had increased to 79 at an accelerated rate. It was found that the human outbreak was linked to diseased pigs and incorrectly diagnosed as Japanese Encephalitis in the beginning. Emergent measures had not been implemented until the pathogen of Nipah virus was correctly identified on 03/19/1999, with the aid from the CDC of USA. Consequently, the outbreak involved 265 human cases with 115 fatalities and culling of more than 1 million pigs [3].

Additionally, sometimes important emergent measures may not be implemented to control a severe outbreak of human or animal infectious diseases, because its pathogen is assumed empirically or legally not that dangerous. The *Streptococcus suis* crisis in 2005 in China is a case in point [5]. It began with a dozen of cases with severe clinical signs from July 12, 2005 to July 21, 2005, in a region in Sichuan province of China. Until July 24, 2005, 58 cases with 17 fatalities were reported and the outbreak was linked to diseased pigs. No evidences about human-to-human transmission were found. Two days later, the pathogen *Streptococcus suis* was identified, and emergent measures such as hygiene education, pig vaccination, proper disposal of dead pigs were implemented. However, culling of the infected or possibly infected pigs have never been implemented, because the pathogen is usually assumed not that dangerous. Consequently, the outbreak extended till to the end of August, 2005 with 157 more cases and 20 more fatalities (China's official website, www.xinhuanet.com).

Therefore, to control a severe outbreak of infectious diseases in time, emergent measures should be implemented as soon as possible, if the outbreak has been known serious, no matter what the pathogen is. This conception sounds rational because some effective emergent measures, such as isolation of patients, movement restriction of close contacts, travel alert and case reporting, extensive disinfection, eliminating the possible infection sources, could be implemented before the identification of the pathogen.

Currently, a method has been formulated to rate the severity of an outbreak of human infectious diseases [6].

This method involves respectively scoring four aspects: the severity of clinical signs, transmission of the infectious disease, case number and the infection source (Table 1). The scores of these four aspects are then multiplied, and the resulted product determines the severity of the outbreak. Higher products represent more serious outbreaks, and the products in the ranges of 1 – 14, 15 – 29, 30 – 59, 60 – 119, 120 – 200, 200 – 450 rate the severity of the outbreak as Grades Ⅰ – Ⅳ, respectively.

Table 1 The scoring of 4 aspects for evaluation of the severity of a human infectious disease outbreak

Aspects	Description	Score
Clinical signs	Mild, usually requiring treatment without hospitalization	2
	Severe, usually requiring hospitalization, but without severe outcomes like death, abortion or body abnormality or malfunction for a long time (e.g. human epididymoorchitis caused by brucellosis)	3
	Severe, <10% cases having developed severe outcomes like death, abortion or body abnormality	4
	Very severe, 10% ~20% cases having developed severe outcomes like death, abortion or body abnormality	5
	Very severe, >20% cases having developed severe outcomes like death, abortion or body abnormality	6
Transmission	No evidences supporting person-to-person transmission	1
	Transmission through special behaviors such as sex is probable	2
	Transmission among frequent close contacts is probable	3
	Transmission among casual close contacts transmission is probable	4
	Transmission among people without close contact is probable	5
Case number	1 – 9	1
	10 – 50	2
	51 – 100	3
	101 – 200	4
	>200	5
Infection source[a]	Without links to domestic animals	1
	With a possible link to domestic animals	2
	With a clear evidence to support domestic animals are infection source	3

Generally speaking, Grades Ⅰ – Ⅳ means that the outbreak is mild, moderate, severe, very severe, too severe and extremely severe, respectively. Totally, there are 375 combinations of the four aspects in this evaluation system, and the evaluation results of these 375 combinations largely support the design idea that higher score products indicate more serious outbreaks.

Using this method the human outbreaks of SARS virus, Nipah virus and *Streptococcus suis* stated above could be rated as Grade Ⅳ (very severe), Grade Ⅲ (severe) and Grade Ⅲ (severe), respectively, at their early stages according to the data given in the introduction part [6].

The method measuring the severity of an animal infectious disease outbreak given herein, involves respectively scoring the following aspects: the direct economic loss due to the outbreak and outbreak control, transmission of the disease, public health significance and novelty (Table 2). The scores of these four aspects are then multiplied, and the resulted product represents the severity of the outbreak. Higher products indicate more serious outbreaks, and the products in the ranges of 1 – 14, 15 – 29, 30 – 59, 60 – 119, 120 – 200, 200 – 450 rate the severity of the outbreak as Grades Ⅰ – Ⅳ, respectively.

Generally speaking, Grades Ⅰ – Ⅳ indicate that the outbreak is mild, moderate, severe, very severe, too severe and extremely severe. Totally, there are 336 ($6 \times 4 \times 7 \times 2$) combinations of the four aspects in this evaluation system, and the evaluation results of these 336 combinations largely support the design idea that higher score products indicate more serious outbreaks.

Using this method, the outbreak of Nipah virus in pigs in Malaysia in 1998, the outbreak of foot and mouth disease in United Kingdom in 2001, the outbreak of *Streptococcus suis* in pigs in Sichuan, China in 2005, the outbreak of African swine fever in pigs in Georgia in 2007, could be all rated as Grade Ⅲ (severe) outbreaks, at certain stages (Table 3). Though these four outbreaks are all rated as Grade Ⅲ outbreaks, the outbreak of Nipah virus was more severe than the outbreak of *Streptococcus suis*, as suggested by their final product scores.

The severity measures should be conducted by a professional entity, like a research institute or the corresponding section of the government, and the rating of a senior entity is more authorative.

Table 2 The scoring of 4 aspects for evaluation of the severity of an animal infectious disease outbreak

Aspects	Description	Score
Direct economic loss due to outbreak and outbreak control	Less than 5,000 USD in the currency value in 2010	1
	5,000 – 50,000 USD in the currency value in 2010	2
	50,000 – 500,000 USD in the currency value in 2010	3
	500,000 – 5,000,000 USD in the currency value in 2010	4
	5,000,000 – 50,000,000 USD in the currency value in 2010	5
	>50,000,000 USD in the currency value in 2010	6
Transmission	No evidences supporting intro-flock transmission	1
	Evidences supporting slow intro-flock transmission (e. g. through sexual behavior), but not rapid intro-flock transmission (e. g. through close contact), have been found.	2
	Evidences supporting rapid intro-flock transmission (e. g. through close contact), or intra-flock transmission through indirect contact, vehicles or vectors, but not through air, have been found.	3
	Evidences supporting intra-flock transmission through air, have been found.	4
Public health significance	No public health significance	1
	Having caused a human outbreak of Grade x severity ($x = 1, 2, \cdots, 6$)	$x + 1$
Novelty	No evidences supporting that the outbreak is new to the country or region have been found	1
	Evidences supporting that the outbreak is new to the country or region have been found	2

Table 3 Evaluation of the severity of four outbreaks of animal infectious diseases at their early stages

Pathogens	Nipah virus [3]	FMD virus[a]	Streptococcus suis [5]	African swine fever [8]
Affected countries	Malaysia	England	China	Georgia
Date for evaluation	March 6, 1999	May 16, 2001	July 24, 2005	July 9, 2007
Score of direct economic loss	3	6	3	5
Score of transmission	3	4	3	3
Score of public health	4	1	4	1
Score of novelty	2	2	1	2
Product of the scores	$3 \times 3 \times 4 \times 2 = 72$	$6 \times 4 \times 1 \times 2 = 48$	$3 \times 3 \times 4 \times 1 = 36$	$5 \times 3 \times 1 \times 2 = 30$
Grade	Ⅲ	Ⅲ	Ⅲ	Ⅲ

[a] From http://www.guardian.co.uk/uk/2001/may/16/footandmouth. comment accessed on 03/ 15 2013.

Moreover, if a human outbreak is rated Grade Ⅲ (severe), it is assumed of a public health emergency of international concern (PHEIC), and should be reported to the World Health Organization (WHO). PHEIC is a key concept of the International Health Regulations adopted in 2005 [7], but its meaning has not been clarified explicitly. Additionally, if an outbreak is rated Grade Ⅲ (severe), it is assumed of an emergency of international concern (EIC), and should be reported to World Animal Health Organization (OIE), although OIE has not adopted such an important concept.

The four aspects involved in the severity rating methods reported herein largely determine the severity of the future development of an outbreak if no emergent measures are implemented to control the outbreak. Crude (not exact) data about these four aspects required by the methods are usually available at the early stage. Therefore, the methods described herein are both rational and practical. The methods reviewed or reported here do not directly consider other aspects including the basic reproduction rate, the generation time, geographical or temporal distribution, etc. These aspects are actually overlapped with the aforementioned four aspects involved in the severity rating methods.

Like the rating of an earthquake in seismology, quantitative rating the severity of an outbreak of infectious diseases should be important in infection control. Unlike the degree of an earthquake, the severity of an outbreak of infectious diseases is dynamic. It may be rated Grade Ⅰ (mild) at the beginning, but when more cases have emerged and more characteristics of the outbreak have been revealed, the outbreak could be re-evaluated to be Grade Ⅱ (moderate) or higher.

The methods reported herein could be optimized in the future, if needed.

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Chimeric Virus-like Particles of Rabbit Hemorrhagic Virus as Carriers for B-cell Epitopes of Foot-and-Mouth Disease Virus

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Summary: In order to obtain the virus-like particles (VLPs) of rabbit hemorrhagic virus (RHDV) displaying B-cell epitopes of foot-and-mouth disease virus (FMDV), the genes of two FMDV B-cell epitopes were fused to the N-terminal, C-terminal and inserted between 306 and 307 aa of the capsid protein. The fused genes were cloned into the donor vector pFastBac™HT A and recombinant baculoviruses were constructed using Bac-to-Bac baculovirus expression system. Six recombinant proteins were expressed effectively in insect cells and confirmed by IFA, SDS-PAGE and Western blot. The immunogenicity of all chimeric VLPs was examined in mice. The results indicated the recombinant proteins could react with both anti-VP60 monoclonal antibody and anti-FMDV polyclonal antibody and could self-assemble into VLPs by Electron microscopy analysis. All chimeric VLPs were able to induce strong VP60-specific and moderate peptide-specific humoral immune responses in the absence of any adjuvants. A moderate level of serum antibody titers detected against B-cell epitopes of FMDV demonstrated the feasibility of RHDV-VLP serving as a presentation carrier for foreign B-cell epitopes.

Introduction

Rabbit hemorrhagic disease (RHD) consists of a single capsid protein with a molecular weight of about 60 kDa (the capsid VP60). The RHDV-VP60 protein, expressed in several heterologous systems, such as *E. coli* (Meyers G et al., 1991), baculovirus-insect cell system (Gelmetti D et al., 1998), yeast expression system (Famos O et al., 2006) and plants (Castanon et al., 2002;) has been shown to provide full protection of rabbits against a lethal challenge by RHDV. FMDV is characterized by at least five cell epitopes, including epitopes from the VP1 141 to 160 (G-H loop) and the C-terminal amino acid residues 200 to 213 (Wong H T et al., 2000). Some researches show that multiepitopes of FMDV could induce a strong immune response (Dimarchi R et al., 1986). Some reports previously have shown that VP60 protein can accommodate insertions of foreign amino acid sequences at the N, C-terminal regions and at a insertion site between amino acid positions 306 and 307 of VP60 protein, without disrupting VLPs formation, raising the possibility of using RHDV-VLPs as foreign epitope carriers for vaccine development (Laurent S et al., 2002; Barcena et al., 2004; Crisci E et al, 2009).

Material and methods

The fusion genes encoding modified capsid proteins were generated by PCR with the primers shown in Table 1. The six recombinant transfer vectors, encoding individual fused proteins described in Fig. 1, were obtain following the instructions of Bac-to-Bac baculovirus expression system. All the resulting bacmids DNA were transfected into monolayer Sf9 cells with lipofectamine 2000 as the manufacturers of the Bac-to-Bac Baculovirus Expression Systems (Invitrogen Ltd). The six recombinant protein were identified by IFA, SDS-PAGE and Western blot. And the chimeric VP60 proteins were analyzed by Electron microscopy.

Table 1 The sequences of primers

Primer	Sequence (5′ to 3′)
VP60-F	5′-TTTGAATTCATGGAGGGCAAAG CCCGCAC-3′
VP60-1R	5′-GCCGTCGACGACATAAGAAAA GCCATTGG-3′
VP60-2R	5′-TTTGTCGACCCCAGAATAACTTG CACTGCCTC-3′
VP60-2F	5′-CACTCTAGAAACAACTCCACCAA CGTGCT-3′
VP60-3R	5′-GCCAAGCTTTCAGACATAAGAA AAGCC-3′
VP60-NF	5′-TTTTCTAGAGAGGGCAAAGCC CGCAC-3′

The recombinant VP60 and the chimeric VP60 constructs were expressed and the self-assembled VLPs

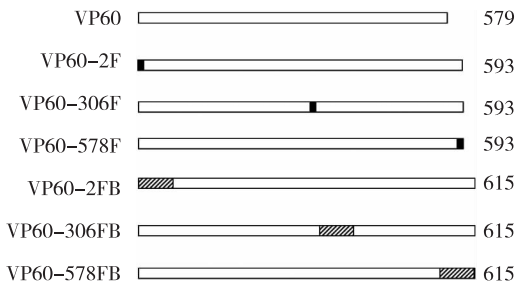

Fig. 1 Six recombinant VP60 constructs (VP60-2F, VP60-306F, VP60-578F, VP60-2FB, VP60-306FB and VP60-578FB) used in this study. The chimeric proteins habour the depicted foreign peptide sequence containing FMDV VP1 derived B cell epitopes at the indicated positions. ■: RHKQEIVAPVKQKL, named F; ▨: GS-RHKQEIVAPVKQKL-GS-VPNLRGDLQVLAQKVART LP-GS, named FB.

were purified by previously described methods (Q. Pan et al., 2008). Male mice (ICR, obtained from Animal Center Laboratory of the University of Yangzhou, China) of 7 – 8 weeks old were randomly divided into fifteen groups with six mice in each group, kept under standard pathogen-free conditions. All mice were inoculated subcutaneously every 2 weeks for 3 times. Group 1, 2, 3, 4, 5, 6 were respectively inoculated with 50 μg of VP60-578F, VP60-306F, VP60-2F, VP60-578FB, VP60-306FB and VP60-2FB VLPs with adjuvants. Group 7, 8, 9 were vaccinated with 50 μg of VP60-2F VLPs, native RHDV-VLPs and 0.2 ml PBS without adjuvants. Group 10, 11, 12 were vaccinated with native RHDV-VLPs, FMDV inactivated vaccine and PBS with adjuvants. Mice were primary immunized with antigen emulsified in Freund's complete adjuvant followed by boosters with antigen in Freund's incomplete adjuvant. Serum samples were collected at 0, 1, 2, 3, 4, 5, 6 weeks post primary immunization. Detection of VP60 specific and Peptide specific antibodies using iELISA. The significance level was established at $P < 0.05$ and all data were analyzed with the SPSS Statistics V17.0 software.

Results and discussion

The cells infected by the recombinant chimeric baculoviruses displayed both VP60-specific (Fig. 2A) and FMDV peptide-specific green fluorescence (Fig. 2B). As SDS-PAGE, all the insect cell extracts infected with recombinant baculoviruses exhibited a major protein band with the expected size of 60 kDa, and was not in wild-type baculovirus infected cells. As expected, the chimeric VP60 constructs containing the FMDV VP1 derived B cell epitopes displayed a slightly slower electrophoretic mobility than the VP60 protein. Monoclonal antibodies directed against RHDV-VP60 protein specifically detected baculovirus expressed VP60 protein as well as the chimeric proteins by Western blot. The chimeric proteins could react with both anti-VP60 monoclonal antibody and anti-FMDV polyclonal antibody. The chimeric VLPs harboring the B cell epitopes were able to self-assemble into VLPs (about 40 nm), and were structurally similar to native RHDV-VLPs (Fig. 3). VP60-specific IgG and peptide-specific IgG antibodies were measured by iELISA at 0, 1, 2, 3, 4, 5 and 6 weeks post primary inoculation. All of the six chimeric proteins could induce strong VP60-specific humoral immune responses and moderate peptide-specific humoral immune responses by means of subcutaneous immunization (Fig. 4A, B) ($P < 0.05$). The ELISA signals exhibited by most of the sera from the groups of mice inoculated with the chimeric VLPs constructions are slightly higher than that corresponding to the groups of mice inoculated with native RHDV-VLPs and PBS (Fig.

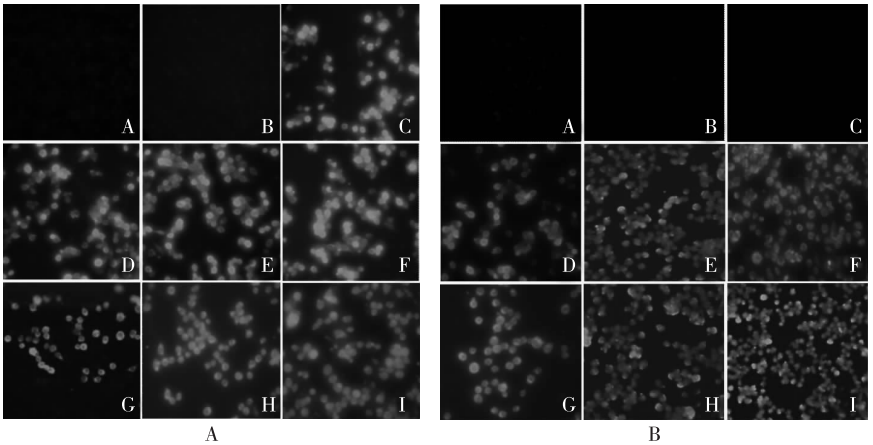

Fig. 2 Detection of the six recombinant proteins by IFA. A, B: (A) Normal cells; (B) Wide-type virus (WT); (C) VP60; (D) VP60-578F; (E) VP60-306F; (F) VP60-2F; (G) VP60-578FB; (H) VP60-306FB; (I) VP60-2FB.

4B). In contrast to other five chimeric proteins, the VP60-2F VLPs induced the highest level of VP60 specific and peptide specific antibodies (Fig. 4A, B) ($P < 0.05$). The results indicated that adjuvants did not affect the levels of VP60-specific and peptide-specific antibodies (Fig. 4C, D) ($P > 0.05$).

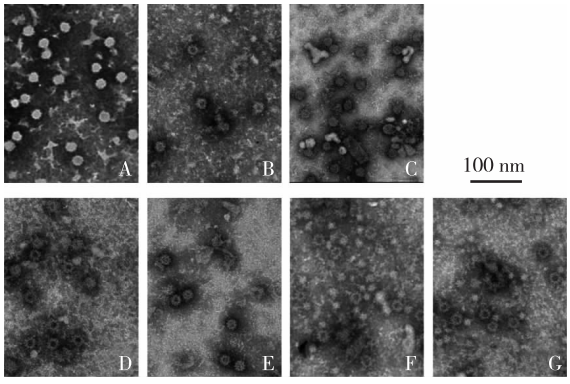

Fig. 3 Electron micrographs of chimeric VP60 particles. Electron microscopy of negatively stained purified chimeric VP60 particles (A), VP60-578F (B), VP60-306F (C), VP60-2F (D), VP60-578FB (E), VP60-306FB (F) and VP60-2FB (G). Scale bar indicateds 100 nm.

Conclusion

The new RHDV-based VLPs might be a suitable system for the induction of humoral responses against foreign B-cell epitopes and could be as multivalent vaccine vector.

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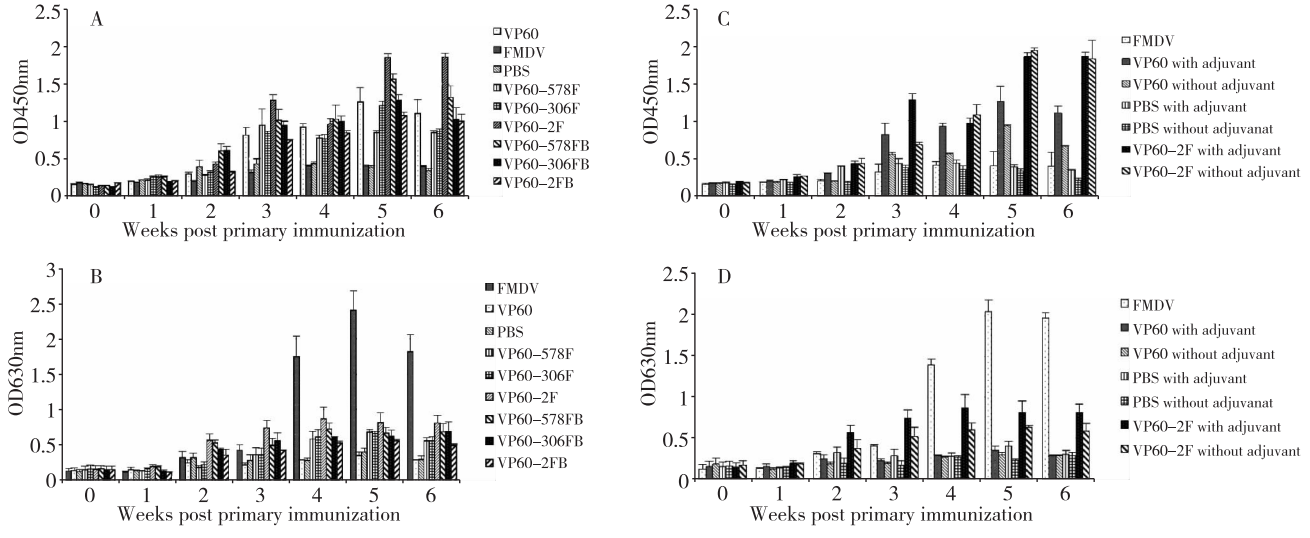

Fig. 4 VP60-specific and peptide-specific humoral immune responses detected by iELISA.

Identification and Genetic Characterization of Bovine Viral Diarrhea Virus Genotype 2 Strains Isolated in Pigs in China

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Summary: Twelve bovine viral diarrhea virus 2 (BVDV-2) strains were screened and isolated from sick pigs with CSF-like symptoms. Homology comparison showed that the E2 genes of the twelve isolates were highly conserved. The full length genome of the one of the BVDV-2 isolates (named as SH-28), which showed a noncytopathic effect in MDBK cell cultures and strong reactivity with MAb Bz-53 raised against BVDV-2, was sequenced. The genome of SH-28 comprises 12,279 nucleotides and contains a large ORF beginning at nucleotide 386 and ending at nucleotide 12073. Genomic comparison and phylogenetic analyses showed that SH-28 fall into BVDV-2 subtype and was most similar to XJ-04 isolate (bovine BVDV-2), but was genetically divergent from ZM-95 (pig BVDV-1).

Introduction

Pig is an important host of pestiviruses, including classical swine fever virus (CSFV), BVDV and bovine border virus (BDV) [1]. Natural infection of pigs with BVDV was first reported in Australia in 1964, but BVDV was not isolated from a naturally infected pig until 1973 [2]. In 1995, a pig BVDV-1 (ZM-95) was first identified in China [3]. The prevalence of BVDV antibodies in pigs in Australia, Ireland, Germany have been estimated at 3% – 40%, and 15% – 20% in Holland [4]. In 2008, an investigation showed that rate of infection of BVDV in pigs in China was 16.3% and all the isolates were BVDV-1 subtypes [5]. So far, there has been no report concerning infection of pigs by BVDV-2 in China. In this study, we isolated and genetically characterized BVDV-2 strains from sick pigs.

Material and methods

Serum samples were collected from sick pigs inoculated with the hog cholera vaccine, which came from a four thousand-piglets piggery with no cattle around in Shanghai. After being inoculated with hog cholera vaccine for a month, approximately eight hundred piglets showed the clinical symptoms of anemia, rough hair coats, diarrhea, and hyperthermia. The course of the infection was quite similar to that described for CSFV infection in pigs.

The samples (the serum and hog cholera vaccine) were inoculated and passaged on MDBK and PK15 cells, respectively. The method of virus isolation had been described previously [6]. RT-PCR primers (Table 1) were designed to differentiate BVDV from CSFV. Besides, an indirect immunofluorescence assay (IFA) was carried out as previous [7].

Table 1 Sequence and location of the RT-PCR primers

Name	The sequence of the primers	Length (bp)
CSFV P1	5'-TC(GA)(AT)CAACCAA(TC)GAGATAGGG-3'	251
CSFV P2	5'-CACAG(CT)CC(AG)AA(TC)CC(AG)AAGTCATC-3'	
FBVDV I-II	5'-CATGCCCATAGTAGGAC-3'	288
BBVDV I-II	5'-CCATGTGCCATGTACAG-3'	
BVDV-1 P1	5'-GGTAGCAACAGTGGTGAG-3'	221
BVDV-1 P2	5'-GTAGCAATACAGTGGGCC-3'	
BVDV-2 P1	5'-CGACACTCCATTAGTTGAGG-3'	117
BVDV-2 P2	5'-GTCCATAACGCCACGAATAG-3'	
BVDV-E2/P1	5'-GGGCTATTGTGGCTGATGC-3'	1175
BVDV-E2/P2	5'-TCTTCAGTATTCACTCCAGCACC-3'	

MDBK cells infected with the isolates without any CPE were harvested to extract total RNA with TRIZOL® LS Reagent (Invitrogen), according to the manufacturer's instructions. The cDNA preparation, DNA cloning and sequencing analysis were done as previous [8,9].

Results and discussion

In this study, 12 BVDV-2 isolates, of which 11 were from the pigs and one was from the hog cholera vaccine, were identified. Given that the 12 isolates have the same genetic characteristics (with strong response to MAb Bz-53, and the E2 genes were highly conserved) and the pigs showed clinical symptoms after inoculation with the vaccine, we speculated that the isolates originated from hog cholera vaccine contaminated with BVDV-2.

The complete genomic sequence of SH-28 (accession number HQ258810) is 12,279 nucleotides and contains a large ORF encoding a polyprotein consisting of 3895 amino acids, which is a new isolate differs from other BVDV strains. After comparison and

analysis of the ORF and individual proteins, SH-28 appeared to be most similar to Chinese bovine BVDV-2 strain XJ-04. In addition, large insertions, which had been reported previously in cytopathic BVDV [10], were not found in SH-28. The splicing sites between the proteins in the ORF of SH-28 were predicted (Fig. 1). Variation was observed in the amino acid sequences flanking the putative protein cleavage sites [11] of the viral structural protein.

		Npro -C	C -Erns	Erns-E1	E1-E2	E2-p7
SH-28	BVDV-2	WVTSC SDEGG	QLVTG ENITQ	FGAYA ASPYC	TGAQG FPECK	QIAMG ARVNT
XJ-04	BVDV-2	WVTSC SDEGG	QLVEG ENITQ	FGAYA ASPYC	TGAQS FPECK	QMAMG ARVNT
890	BVDV-2	WVTSC SDEGS	QLVTG ENITQ	FGAHA ASPYC	TGVQG FPECK	QMAMG AGVNT
NADL	BVDV-1	WVTTC SDTKE	QVTMG ENITQ	FGAYA ASPYC	TGVQG KKLFD	QKALG IQYGS
ZM-95	BVDV-1	WVTSC SDTKG	QVTAG ENITQ	FGAYA LSPYC	TGAQG YPDCK	QRASG TQCGA

		p7-NS2-3	NS2-3-NS4A	NS4A-NS4B	NS4B-NS5A	NS5A-NS5B
SH-28	BVDV-2	GVVKA SKTNT	QVTGL STAEN	ELKEL QKIKE	KIRNL SSNYL	YTMKL SSWST
XJ-04	BVDV-2	GLVKA REINT	QVTGL STAEN	ELKEL AVGDL	KIRNL SGNYI	YTMKL SSWST
890	BVDV-2	GAVKA IPPEE	QVTGL STAEN	ELKEL AVGDL	KIRNL SGNYI	YTMKL SSWST
NADL	BVDV-1	DVVKA DSGGQ	QVTGL SSAEN	ELKEL ASGDV	KIRNL SGNYI	YAMKL SSWFL
ZM-95	BVDV-1	GAVKA ESGTQ	QVAGL SSAEN	ELKEL ALGDV	KIRNL SGNYI	YTMKL SSWFM

Fig. 1 Cleavage sites between structural and nonstructural proteins of SH-28, and BVDV-1 and BVDV-2 reference strains

Conclusions

To date, only two complete genomic sequence of pig BVDV-1 (ZM-95 and SD0806) are available and the identities between SH-28 and ZM-95 or SD0806 were low, which implied that there is considerable genetic divergence between different pig-source isolates. Usually, it is assumed that pig origin BVDV is derived from cattle origin BVDV [12]. This study provides important information concerning the genetic relationship between cattle origin and pig origin BVDV. In addition, it enriches the available genomic sequences and exerts a positive influence on research into the genetic evolution of pig-origin BVDV.

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Distribution of Estrus in Hair Ewe during the Season of Low Activity Reproductive Conditions of Northwestern Mexico

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Summary: The aim of this study was to determine the presence and duration of estrus in ewes Pelibuey environmental conditions in northwestern Mexico with constant feeding. The study was conducted in the Valley of Mexicali, Baja California, located at 32° NL with arid climate. This study will last for one year (January to December), 24 adult sheep were used to measure multiparous Pelibuey estrous changes for a year, under constant feeding. To measure estrous activity, we used two studs, that were introduced twice a day for half an hour each time the sheepfold, females were in heat detected by the male. The month with the highest percentage of females in heat was March (86.36%), April (81.8%), in February and June showed zeal and 77.3% in January (50%) showing significant difference ($P < 0.05$). For the variable duration of estrus were the months of June and March with 38.9%, 35.3% and 26.3% respectively, in January the lowest rate that jealousy between 24 – 36 hours presented with a 20.0%. Was created a completely randomly. Variables expressed as percentages were analyzed with chi-square, continuous variables with analysis of variance. SAS 2004.

Introduction

Sheep characteristically present a seasonal reproductive activity throughout the year, at the time being predominantly short days (autumn-winter) when this species is regularly estrous activity (Cerna et al., 2000). In this sense, the photoperiod is the main factor that regulates the reproductive activity of sheep, and indirectly also affect other factors, such as food availability, temperature and humidity. The phenomenon of seasonality is most marked in wool breeds than in hair breeds. Studies in tropical conditions (Gonz lez-Reyna et al., 1992) and temperate (Arroyo et al., 2007) Pelibuey of Mexico, have shown that hair sheep are characterized by present reproductive activity throughout the year, although a decrease in the presence of ewes in estrus were observed between the months of January to April, without considering a time of deep anestrus and in the wool. In northwestern Mexico, bred sheep hair, as *Pelibuey* and D*orper*, are preferred by farmers because of their high reproductive capacity and adaptation to climatic extremes that dominate the region (up to 50℃ in summer and below 0℃ in winter). However, estrous behavior throughout the year has not been evaluated. Correa-Calderón et al. (1996) found that the goats had two periods of anoestrus, the first in the months of April-May, and a second in the summer, which correlated with photoperiod and heat stress, respectively. Therefore, it is necessary to know the estrous behavior of hair sheep in conditions of northwestern Mexico, which will be the basis for the generation of other knowledge in this field of sheep production. The aim of this study was to evaluate estrous behavior during the period of low reproductive activity of hair sheep under environmental conditions in northwestern Mexico.

Material and methods

The experiment was conducted from January to June at the Zootechnical Institute of Agricultural Sciences, UABC, which is located in the Valley of Mexicali, Baja California. We used 22 multiparous females Pelibuey aged between 3 and 4 years, and body condition between 3.5 and 3.0 points on a scale of 1 to 5. The sheep were kept in a single group within a pen provided shade, watering and feeding. Each day, the sheep were fed a diet consisting of wheat straw, alfalfa hay and mineral mixture, which was formulated based on the nutritional requirements of maintenance. Both food and water were provided *ad libitum*. To determine the presence of estrus, apron fitted two males were introduced in a staggered daily (twice daily, 8:00 and 18:00 h) to corral females for detection of estrus. It was felt that a female was in estrus if kept static when mating. Females showing signs of estrus were removed to an adjacent yard and were recorded date, time and identification. The percentage of females in estrus was determined monthly. The duration of estrus of these sheep was classified into four categories (<12 h, 12 – 24 h, 24 – 36 h and > 36 h) and compared the percentage of ewes in estrus between categories within each month. Data were analyzed with the chi-square test, using PROC FREQ of SAS (2004).

Results and discussion

Fig. 1 shows the distribution of estrus presence of sheep Pelibuey from January to June. The percentage of ewes in estrus was similar ($P > 0.05$) in the months of January and May, but in recent months, the sheep had lower ($P < 0.05$) percentage of estrus in the months of February, March, April and June. This is contrary to what was reported by Arroyo et al, 2007 who found a similar distribution of jealousy is an experiment in the east. Fig. 2 shows the distribution of sheep in estrus based on its duration evaluated in different months. The percentage of ewes in estrus lasting < 12 h or $24 - 36$ h, was similar ($P > 0.05$) between the months of study. However, a higher ($P < 0.05$) percentage of ewes in estrus $12 - 24$ h in the months of January, February and May compared to the months of March, April and June. The percentage of ewes in estrus after 36 h also varied ($P < 0.05$) in the month, being in the months of March, April and June higher and lower in the months of January, February and May. This distribution is equal to that reported by Navarro and Torres in 1985, which found that 86% of the sheep studied showed jealousy between $24 - 36$ h, and this happened during the months of study, without presenting a significant difference.

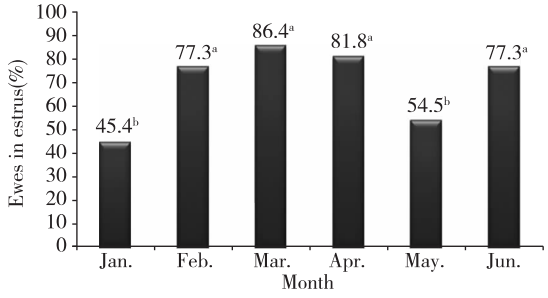

Fig. 1 Presence Pelibuey estrus in ewes during the time of low reproductive activity in Northwest Mexico
Unlike literal represents statistical difference $P < 0.05$.

Conclusions

The distribution of estrus in sheep pelibuey Mexicali Valley is not the same to that presented in other regions of the country, but the duration of these and their distribution in the months studied has a behavior similar to those reported in other regions.

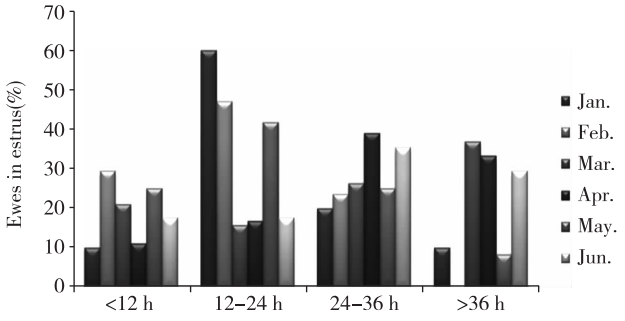

Fig. 2 Distribution by month and duration of estrus in ewes during the time Pelibuey low reproductive activity in Northwest Mexico

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Activation of Physiological Thermoregulation Mechanisms in Hair Ewes by Effect of Heat Load Acquired during Summer

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Summary: Ten non pregnant Katahdin × Pelibuey multiparous ewes were used to evaluate the effect of accumulated heat load during summer on the activation of physiologic thermoregulatory mechanisms in an arid-dry environment. Ewes were allocated in a penduring the summer of 2011. On day 15 of each month, live weight (LW), body condition score (BCS), rectal temperatures (RT), respiration rate (RR) and glucose concentration were recorded during the morning and afternoon. Mean comparisons were performed among months and times. The LW was similar ($P > 0.05$) among summer months, but BCS increased ($P < 0.05$) in September compared with the other months. In August, RT was lower ($P < 0.05$) during the morning and higher ($P < 0.05$) during the afternoon compared with the other months. During the morning, RR in July was similar ($P > 0.05$) to that of September, but greater ($P < 0.05$) to those from June and August. Compared with other months, lower glucose concentration was observed in August during the morning and during the afternoon in September ($P < 0.05$).

Introduction

In extreme climates as they exist in the arid north of Mexico, presents high summer temperatures causing heat stress to pets living there. Among the compensatory mechanisms that are activated are the first physiological type such as increased respiratory rate, panting or sweating (Abdel-Hafez, 2002). In arid regions of the country, temperatures prevailing high (between 30 and 50℃) during the four summer months (June to September), with slight diurnal variations in ambient temperature, which prevents sheep lose heat load night. It is known that the cumulative heat load while the ambient conditions of hyperthermia, and once they change to thermo neutral, releasing heat that accumulated charge may take several weeks, depending on the time of exposure to high temperatures (Cain et al., 2006). Therefore, the aim of this study was to evaluate the effect of accumulated heat load through the summer on the activation of physiological thermoregulatory mechanisms of hair sheep in an arid and dry.

Material and methods

The study was conducted from June to September 2011 at the Experimental Sheep Institute of Agricultural Sciences of the UABC, located in the Valley of Mexicali, Baja California. 10 females were used multiparous females Katahdin × Pelibuey, between 3 and 4 years, with average PV 53.6 ± 4.15 kg and average CC of 3.26 ± 0.30 units (scale of 1 = very thin to 5 = very fat). The sheep were kept in a pen with a dirt floor (3.7 m^2/animal), provided shade (2 m^2/animal) feeders and fountains. Feeding during this period consisted of a diet of alfalfa hay (50%) and wheat straw (50%) *ad libitum*. Water is offered *ad libitum*. On the 15th of each month, the sheep were individually weighed and body condition was measured before offering the food of the day. On the same day at times 6:00 and 18:00 h, blood was sampled and the FR was registered (count intercostal number of movements per minute) and TR (with digital thermometer). Blood samples were collected in 6 ml vacutainer tubes by jugular venipuncture and centrifuged at 3500 rpm for 10 min at 10℃. The serum obtained was placed in 2 mL aliquots and stored at −20℃ until used for determining the glucose concentration by radioimmunoassay method reader blood chemistry (EK TACHEM DT60 II® System Johnson & Johnson). Additionally, data from ambient temperature (T) and relative humidity (RH) is requested to UABC Weather Station. From this information we calculated the temperature-humidity index (THI) with the equation proposed by Marai (2001): $THI = T - \{[0.31 \text{ to } 0.31 * HR)] * (T - 14.4)$. In this scale a THI to 22.2 indicates more heat stress conditions for animals. Las variables were subjected to analysis of variance using a completely randomized design where the model included month and time fixed effects. Comparisons of means were made with "t" test to $\alpha = 0.05$. All statistical analyzes were performed using the SAS statistical package (2004).

Results and discussion

The average THI T obtained in the present study were to 32.0℃ and 27.7 units in June, July and 31.4 units 35.9℃, 36.6℃ and 31.8 units in August, September and 34.3℃ and 30.2 units. These climatic conditions of heat stress were considered for the sheep, July and August being the most critical months since the T was higher. In general, as evidenced sheep production systems during the summer are in an environment of heat stress for prolonged periods of time, which causes the sheep have to activate compensatory mechanisms of acclimatization, and later adaptation (Marai et al., 2007). In the present study, the PV was not significantly different ($P > 0.05$) through the summer months, while the CC was similar ($P > 0.05$) between the months of June, July and August, increasing significantly ($P < 0.05$) in September compared to other summer months (Fig. 1). These results indicate that haired breeds are highly adaptable, and physiological thermoregulatory mechanisms activated were efficient enough to keep the sheep in homoeothermic conditions, without the need to alter the biological functions such as food and water consumption (Abdel-Sameel, 1991). That has not been affected animal's biological functions led to the PV and CC not go down during the summer. In contrast, the CC was increased in late summer, suggesting that the energy metabolism of hair sheep is still fine, no effects of a possible cumulative heat load acquired through summer. In Table 1 results shows DETR, FR and blood glucose concentration as a result of the summer months. In August, the TR in the morning was lower ($P < 0.05$, 38.4℃) and in the afternoon higher ($P < 0.05$, 39.2℃) compared with the other months, including the TR did not change significantly ($P > 0.05$, on average 38.6℃ in the morning and 38.9℃ in the afternoon for June, July and September).

Marai et al. (2007) indicate that the TR thermo neutral conditions vary from 38.3 to 39.9℃ for sheep. Under heat stress conditions that prevailed throughout the summer, the TR morning and afternoon in the studied sheep remained in the range indicated by Marai et al. (2007). Added to this, possibly sheep in August reduced their TR modifying behavior to avoid metabolic heat production at night, enabling them to have greater heat loss (Cain et al., 2006). Moreover, being a highly efficient to lose body heat at night, in the month where high ambient temperatures were higher may be an adaptive mechanism of hair sheep to support the load of body heat to accumulate throughout the day, since in this study also found that in August TR appeared higher in the afternoon than in the months of June, July and September. Most TR during late August was attributed to higher T recorded in that month (37℃) compared to the other months. Furthermore, the inability of physiological compensatory mechanisms (as FR) to dissipate the heat load was accumulated throughout the day. Moreover, in the morning, the FR was higher ($P < 0.05$) in July than in June and August. The FR observed in September was similar ($P > 0.05$) for June and August, and July. In the afternoon, the FR was higher ($P < 0.05$) in August than in other months of summer, but the FR in July was lower ($P < 0.05$) than that recorded in June and September. In general, the FRs were lower in the morning than in the afternoon, which is consistent with

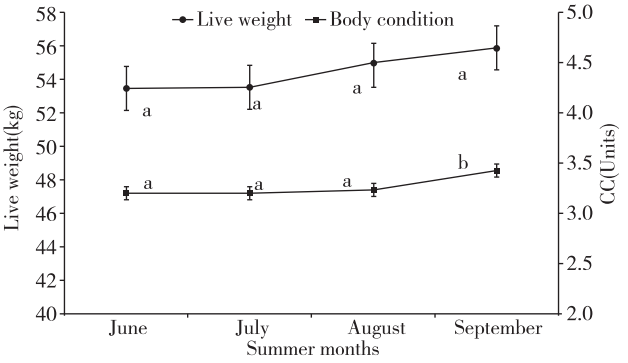

Fig. 1 Live weight and body condition (BC) of hair sheep during the summer months. (a, b indicate significant differences between months $P < 0.05$).

Table 1 Respiratory rate, rectal temperature and glucose concentration of charged hair sheep during summer heat

	Summer time				E. E.
	June	July	August	September	
Rectal temperature (℃)					
AM	38.6[a]	38.6[a]	38.4[b]	38.6[a]	0.07
PM	38.9[a]	38.9	39.2[b]	39.0[b]	0.07
Respiratory (rpm)					
AM	39.2[a]	48.0[b]	39.6[a]	44.9[ab]	3.0
PM	110.2[a]	98.8[b]	117.6[c]	111.2[ab]	2.6
Glucose (mg/dL)					
AM	55.2[a]	62.8[b]	47.7[c]	57.5[a]	1.5
PM	58.6[a]	65.8[b]	58.2[a]	50.6[c]	2.7

[a,b,c] different letters within a row indicates differences $P < 0.05$.

that reported by Marai et al. (2007), and is associated with lower T recorded in the mornings. Furthermore, we observed higher FR during August afternoons compared with the other months because the T was also higher at this time of day (16:00 h), although the mechanism of thermoregulation was not enough to keep the sheep in homoeothermic, so that the TR is increased. It is also noted that FR fluctuated during the months later, on July lower intermediate June and September and August higher. This is probably an adaptation mechanism to maintain homeostasis when subjected to prolonged periods of hyperthermia. In this way, the sheep can reduce water loss by evaporation body (Cain et al., 2006).

The glucose concentration was lower ($P < 0.05$) in August than in other months of study in the morning. This result may be due to a reduction in food consumption at night, considering that month was the hottest, and as previously mentioned, sheep lost body heat accumulation acquired on day modifying its behavior (less movement at night). Afternoon, the glucose concentration was greater in July ($P < 0.05$) and lower ($P < 0.05$) in September. The highest concentration of glucose in July was due to the FR was lower compared to the other months, at least in the afternoon. Energy expenditure by the activation of this mechanism is higher than other thermoregulatory mechanisms (Marai et al., 2007).

Conclusions

In conclusion, hair ewes showed great adaptation to the high temperatures observed during summer, and it was noted that their body temperature was regulated through variations in the RR without altering physiological functions, such as BW and BCS.

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Dissecting Genetic Resistance to be an Alternative for the Chemical Therapeutics to Treat Parasitic Infestations of Food Animals

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Summary: Breeding for resistance to gastrointestinal nematodes in sheep has the potential to minimize the cost of anthelmintics, avoid reduced animal productivity, prevent drug resistance and allay public anxiety about the increasing use of chemicals in the food processing. There are many trials to identify QTLs or genes for resistance to the gastrointestinal nematodes which allow for resistance selection without the expensive and wasteful animal testing of nematode infection but the real challenge is that many different types of parasites may not be susceptible to the same immune pathways and hence, the identification of certain QTLs or genes requires extensive phenotypic studies.
Key words: QTLs, MHC, DQB1, genetic resistance

Introduction

Early in the 1960s, Thiabendazole and Levamisol were the first broad spectrum anthelmintics; the massive use of these broad spectrum anthelmintics had a revolutionary effect on sheep breeding industry. Many types of these drugs have been available since that time. The sheep herds are commonly infested by wide varieties of gastrointestinal nematodes and more than ten different species can occur in one infested sheep (Armour and Coop, 1991a). Therefore, the broad spectrum anthelmintics are chosen according to its efficacy against a wide range of adult parasites or their developing stages.

Candidate gene studies form the basic approach upon which most of our understanding of the possible mechanisms of anthelmintic resistance depends. This is done by making a guess about which genes might be involved in this mechanism and then testing the hypothesis by designing and conducting experimental work. The main themes of these experiments are to find a linkage between some of the resistance traits and the candidate genes, this is done by conducting the research and making comparison between the susceptible and resistance field populations as a field study or artificially selecting for resistance during experimental infections, one of the most prominent successes of this approach has been determination of the isotype-1 β-tubulin locus as a clear marker of benzimidazole resistance in Haemonchus contortus (Gilleard, 2006).

Material and methods

Blood was collected by jugular vein puncture into evacuated glass tubes containing the anticoagulant disodium EDTA (Becton Dickinson UK Ltd, Oxford, UK). Faecal samples were taken directly from the rectum of the lambs and a modified McMaster salt flotation technique (Wells, 1963) was performed to estimate the concentration of nematode eggs in the faeces.

The assays for IgE and IgA specific antibodies to L3 and L4 stage were performed as described previously (Pettit et al., 2005), employing the muse monoclonal anti-ovine IgE, clone 2F1, and anti-ovine IgA (Serotec, Oxford, UK). Detection was with the goat anti-mouse HRP (Dako).

DNA was extracted from blood leucocytes by using the QIAamp Blood Maxi Kit (Spin Protocol). PCR products of MHC class II genes (DQA1, DQA2, DQB1 and DQB2) were performed using sheep genomic DNA, *Taq* polymerase (Qiagen) and Master Mix (MM) solution. PCR master mix was prepared in a PCR tubes on ice; samples were placed in a thermocycler (Gene Amp-PCR system 2700 Version 2.0-Bio systems A&B). The PCR conditions were adjusted according to each gene after optimization trials to reach the best melting temperature.

Results

The association between MHC class II genes and faecal egg counts in Scottish Blackface sheep:

Fig. 1A, B and C illustrate the effect of DQB1 alleles on faecal egg counts at 3, 4 and 5 month age respectively. There was a highly significant effect of the DQB1 allele (AJ238939) at 5 month age ($P = 0.0176$), (Fig. 1C), also there was a possible effect of the DQB1 allele (AJ238945) at 4 months of age ($P = 0.0709$), (Fig. 1B), and there was no significant effect of the DQB1 alleles at 3 month age.

Fig. 2A, B and C illustrate the effect of DQB2 alleles on faecal egg counts at 3, 4 and 5 month age

respectively. There was a very highly significant effect of the DQB2 allele (Newallele6) at 3 (P = 0.0186) and 4 months of age (P = 0.0229). Also there was highly significant effect of the DQB2 alleles (AJ238932 and Newallele7) at 5 months (P = 0.0149) and (P = 0.02) respectively. There was a possible effect of the DQB2 alleles (AJ238937 and U07030)) at 3 months (P = 0.082), and 5 months (P = 0.0747) respectively.

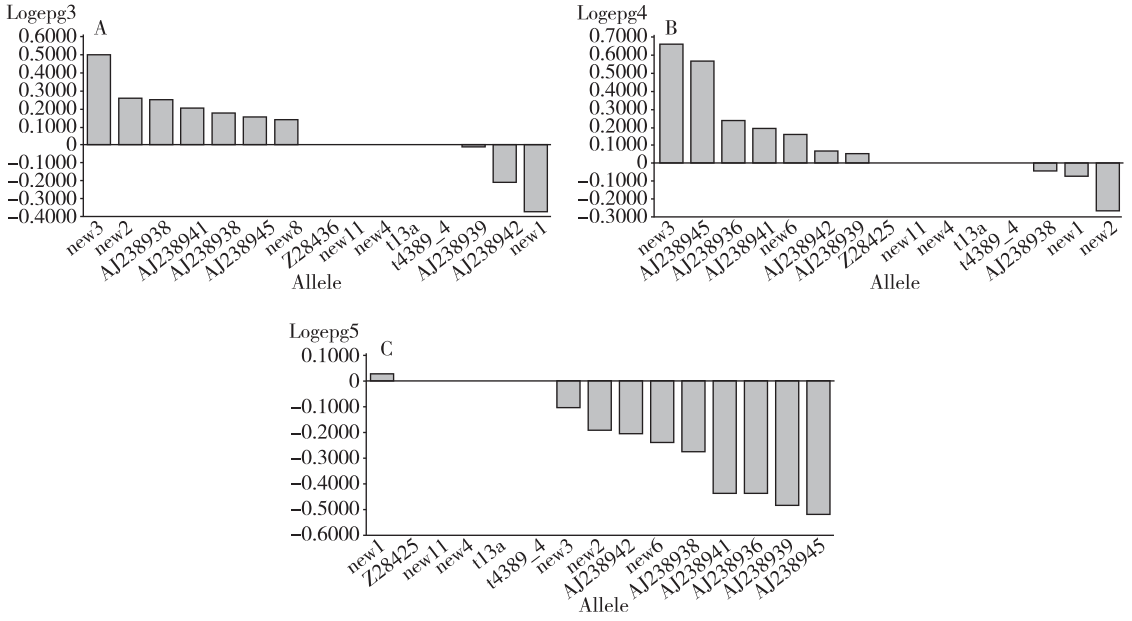

Fig. 1 Association between DQB1 alleles and log FEC in A – 3 months, B – 4 months and C – 5 months in Scottish Blackface rams

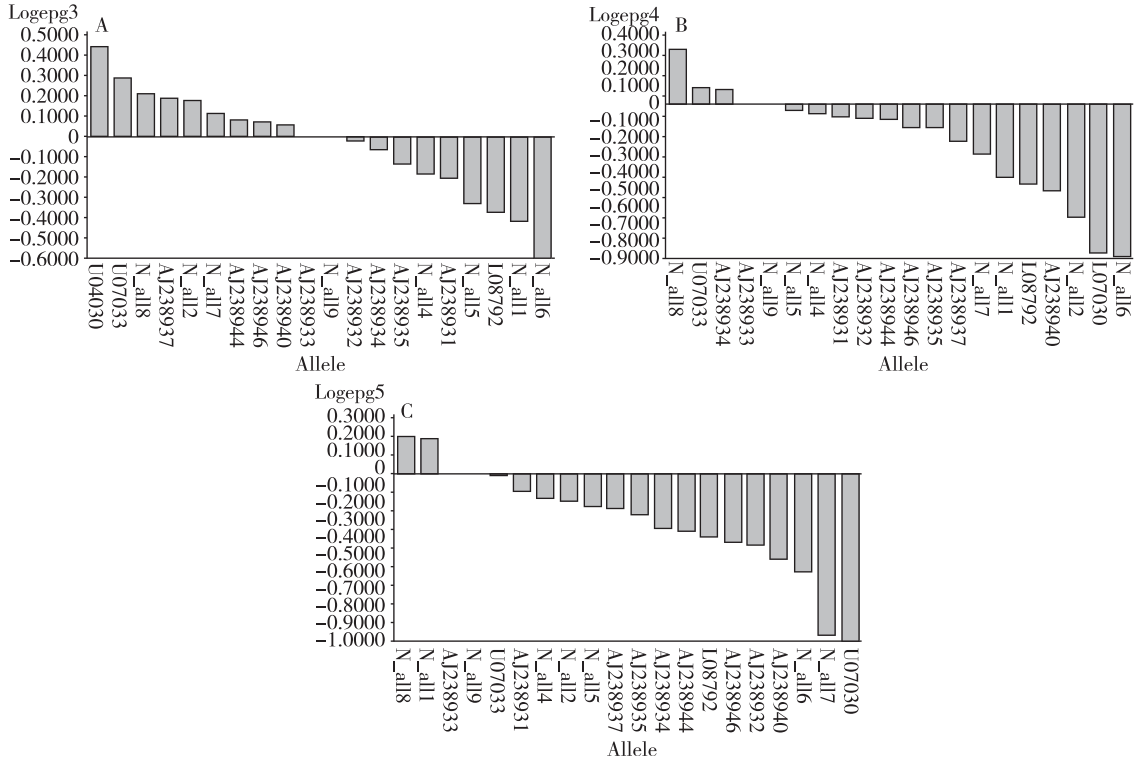

Fig. 2 Association between DQB2 alleles and log FEC in A – 3 month, B – 4 month and C – 5 month in Scottish Blackface rams

Disscusion

The results show variable associations between the different alleles of the two loci DQB1 and DQB2 with the faecal egg counts in Scottish Black Face at 3, 4 and 5 months of age.

The association between the MHC and resistance to nematode infection has been reported for several species including cattle (Takeshima et al., 2008), mice (Else et al., 1990) and sheep (Schwaiger et al., 1996; Stear et al., 2007).

The obtained results of association between some of the alleles at DQB1 and DQB2 loci and the Faecal egg counts in Scottish Blackface rams coincide with the results obtained at another locus belonging to The Ovar-MHC class II region (DRB1); The DRB1 locus codes for protein within the binding area to antigens on macrophages and lymphocytes B (Anderson and Rask, 1988), shows high level of polymorphism which associated with sheep resistance to the gastrointestinal nematodes (Schwaiger et al., 1996).

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The Genetic Background for Need of Partus Induction and Birth Assistance in Piglet Production

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Summary: The aim of the current investigation was to examine the genetic background for partus induction (PI) and birth assistance (BA) in piglet production. Both, PI and BA, are the most important farrowing related management interventions. They are often used to improve the efficiency of farrowing within working time and to optimize the number of piglets born alive, and the number of weaned pigletsas well. A data set of 1,680 sows was analyzed regarding PI and BA in 2,001 litters. A genome-wide association study was conducted in order to find associations between the phenotypes PI or BA and single nucleotide polymorphisms (SNPs). For this purpose, 596 sows were selected for high-plex genotyping using the Illumina PorcineSNP60 BeadChip. Moderately significant SNPs were found for both phenotypes. In addition, sows with hormonal PI showed an about twice-fold risk for the need for BA. Further, the need for BAgoes along with a higher risk of Postpartum Dysgalactia Syndrome(PDS).

Introduction

Because modern piglet production usually aims at large homogenous batches of piglets, the synchronization of farrowing is accomplished through hormonal partus induction (PI). Several studies showed a decrease in the number of stillborn piglets in hormonally initiated farrowing compared to spontaneous farrowing (Bardehle et al., 2012; Roost et al., 1986). Besides PI, the focus of this study was laid on birth assistance (BA) which is applied in case of dystocia. Dystocia is characterized by an extended partus interval between neonates, and by abnormal birth positions. With extended partus intervals, parturition length is increasing, and Holm et al. (2004) verified a high genetic correlation between duration of parturition and BA.

Material and methods

In this study, relevant traits were recorded for 1,680 sows. The data was assessed on six farms with similar housing and management conditions. Sows originated genetically from lines of the Pig Improvement Company (PIC). In total, data from 12 lines was available including two purebred lines: Large White (LW) and Landrace (L). The other lines were different crossbreeds from L, LW and Duroc (Du). The on-farm sampling took place between 04/2008 and 10/2010. Performance parameters, the application of PI, and the need for BA were recorded as well. For further analysis, the parameters PI, BA and PDS were treatedas binary traits. In total, 2,001 litters were recordedfrom 1,680 sows, with 268 sows (16%) contributing more than one farrowing. Sow parities ranged from first to 11th parturition. In 15.3%, sows were primiparous. A detailed overview of the distribution of parity numbers is shown in Table 1.

Table 1 Distribution of parity numbers (2,001 litters)

	Parity											Σ
	1	2	3	4	5	6	7	8	9	10	11	
Frequency	306	405	366	319	240	192	107	55	6	4	1	2,001
Percentage	15.3	20.2	18.3	15.9	12.0	9.6	5.3	2.7	0.3	0.2	<0.1	100

Based on the collected data, 597 individuals were selected for genotyping using the commercially available PorcineSNP60 BeadChip from Illumina. This chip contains 62,163 SNPs, including 1,550 sex-linked and 60,613 autosomal SNPs. The SNP map positions are based on genome assembly Sscrofa10.2 (Ensembl v67, www. ensembl. org) and mapping data was used as provided by MartienGroenen on www. animalgenome. org/repository/pig/Genome_build_10.2_mappings/.

The analysis of the phenotypic and genomic data was

conducted using the GenABEL package (Aulchenko et al., 2007). Quality control was implemented at two levels. In a first step, samples were excluded in case of underrepresented lines. For further analysis, every farm and line had to feature individuals for both states of the binary traits PI and BA. Furthermore, the steps of quality control of the genotypic data included thresholds for the SNP-wise call rate of 0.9, the call rate per individual of 0.95, and a minor allele frequency of 0.05. For the verification of genome-wide associations, the Bonferroni-corrected p-value was specified as 1e-06. In summary, 578 sows and 49,709 SNPs were left for the analysis of genome-wide association. In addition, heritability was calculated using a polygenic model for binary traits within GenABEL.

Results

In general, the results of the phenotypic analysis of all 1,680 (A) sows as well as from the 578 sows after quality control (B) showed the same tendencies. The proportions of sows without any intervening action (PI or BA) were 34.2% (A) and 36.9% (B), respectively. In about 18% of the investigated sows (17.4% (A) and 18.3% (B)), both PI and BA were performed. In 48.3% (A) and 44.8% (B) of the sows PI or BA were exclusively applied, respectively. The odds ratios for BA after PI were 2.11 (A) and 2.13 (B) in comparison to sows without PI. Whereas BA increased the risk (odds ratio = 1.9) for the occurrence of PDS, no relation between the application of PI and the subsequent occurrence of PDS was observed. The application of PI differed significantly between lines and farms, whereas BA differed only in tendency. The mean percentage of sows with BA over the five lines was (27.2 ± 6.4)%. The clear difference between the percentage of sows with BA of the two purebred lines L and LW was not reflected in their crossbreeds. From a farm-wise view, an average of (22.2 ± 8.7)% sows needed BA. This percentage differed between the farms from 7.7% to 29.9% of sows with BA.

The heritabilities for the traits PI and BA were 0.26 and 0.10, respectively. Accomplishing the genome-wide association analysis, both parameters showed moderately significant genetic associations (Table 2).

Regarding the five most associated SNPs (top 5), it was noticeable, that most of them for PI were located on porcine chromosome (SSC) 3 (five SNPsin top 12). The PI associated SNP ALGA0122211 (SSC3) is located within the ataxin 2-like gene (*ATXN2-L*). Currently the function of the *ATXN2-L* gene is unknown. With respect to BA, the associated SNPs were located on SSC7 (nine SNPs in top 16, and eight SNPs in top 12). For the other associated SNPs, no genes were described, but several genes are located within 5 to 500 kbp next to these associated SNPs.

Table 2 The five most associated SNPs for PI and BA with their chromosomal positions (SSC:map position), their reference number (rs number) and the respective p-value.

Parameter	SNP-name	SSC:map position	Rs number	Allele 1	Allele 2	Pc1df
PI	MARC0014182	18:46330470	rs81283831	G	A	1.35E-05
	ALGA0122211	3:18697815	rs81311628	C	A	7.17E-05
	H3GA0008948	3:17859317	rs81377082	G	A	0.0001
	H3GA0008948	3:17859317	rs81377082	G	A	0.0001
	ALGA0059633	10:64805716	rs81426495	A	G	0.0001
BA	MARC0001735	7:88483539	rs80994818	A	G	1.05E-05
	ALGA0042987	7:88557851	rs80845197	A	G	1.05E-05
	ALGA0042968	7:88215937	rs80880794	G	A	2.39E-05
	MARC0028461	12:53521694	rs81223573	C	A	4.46E-05
	ALGA0043002	7:88886555	rs80990618	C	A	9.56E-05

Discussion

In summary, the significant coherence between line and PI or BA, respectively, as well as the low to moderate heritability emphasizes the genetic background for these traits. Berg et al. (2001) accounted depending on line a comparable heritability for BA from 0.04 to 0.11. In fact, the implementation of PI depends mostly on management conditions on farm, but there is still an evidently genetic part when consideringits heritability of 0.26. The top five most related SNP to the parameter PI were located on SSC 3, 10 and 18. On these chromosomes, Onteru et al. (2012) found candidate regions for the parameters gestation length in the second and third parity. However, we could not confirm highlighted genes of this study (Onteru et al., 2012).

Contrary to the current study which did not prove any relations between the implementation of PI and following puerperal diseases such as PDS, Roost et al. (1986) found a decrease of puerperal diseases with 11.3% affected sows with PI, in comparison to 20.7% affected sows in the control group without PI. However, in our study the probability for the need of BA doubled in case of prior PI. This increased risk for BA after

implementation of PI was confirmed by Lucia et al. (2002), who indicated an association between oxytocin use and a higher frequency of vaginal palpations.

Conclusions

This study found relevant genetic loci for the birth management traits PI and BA. The associations between phenotypes and genotypes were merely moderately significant, however, these traits are very complex and do not only depend on genetics. Although BA is applied to prevent neonatal losses, an influence of BA on the onset of PDS was shown by the datasets. Regarding animal health and the economic consequences of PDS, the implementation of BA contains a lot of risks. From an animal welfare point of view, the implementation of BA cannot be denied to sows who need it, however, the aim should be to breed against the necessity of BA. If required, BA should be given under the best hygienic conditions as possible. Further studies should deal with more detailed qualitative or semi-quantitative phenotypes than the binary classification used in this first approach.

Acknowledgement

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Skin and Subcutaneous Lesions Induced by Application of Para-phenylenediamine in Rabbits

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Summary: para-phenylenediamine (PPD) is a black dye used in permanent oxidative hair dyes. It is a major cause of allergic contact dermatitis. So these studies were designed to confirm this effect on the skin. 18 adult domestic rabbits were divided into three groups, first group used as control, second group painted with PPD in a conc. of 2%. Third group painted with PPD (2%) and administered antox (2 mg/animal/day) in the diet as antioxidant. PPD applied twice weekly for 6 months. The results were areas of alopecia, necrosis of skin, sensitization of skin (patchy hyperemia). Histopathological sections revealed sever dermatitis with infilteration of the dermis with a great number of eosinophils, Folliculitis and perifollculitis were also seen. Tubulonephritis, myocarditis and rhabdomyolysis was also reported. leukocytosis with prominent eosinophilia in the blood. In the third group antox did not protect the skin of rabbits from the deleterious toxic effect of PPD. From these results we can concluded that PPD was associated with allergic contact dermatitis, its toxic effect extend to circulation and internal organs so strict control of its practical uses should be adopted.
Key words: para-phenylenediamine, allergic contact dermatitis, rabbits

Introduction

Para-phenylenediamine (PPD) is one of cosmetics that used as permanent hair dye that many of consumers use it to improve their appearance. Take in consideration, the frequency of using hair coloring products, the ingredients of these products must be safe [1]. Human skin is permeable for the substances applied topically; these cutaneous features may aid the absorption and penetration of applied substances involving those of hair dyes [2]. It is a major cause of allergic contact dermatitis (ACD). Most of data report a damaging effects of a commercially hair dye on the skin of rabbits, where the exposed areas were turned black, dried and easily detached from skin. Irregular areas of alopecia and scabs were noted on the back region. Area of skin sensitization were seen on the back region. This sensitized area was hyperaemic and alopecic (devoid from hair) with excess crusts found on it [2]. Other studies report changes in the blood chemistry after hair dye application, there was an increased serum level of alkaline phosphtase, ALT, AST, GGT, amylase, cholesterol, triglyceride, billirubin, creatinie and urea. Moreover a decreased serum level of glucose and potassium were also seen. And at all time intervals the plasma proteins concentration was decreased.

Material and methods

18 adult domestic rabbits aging 2 months were divided into three groups:

- Group one used as control.
- Group two (six rabbits) painted with PPD (was purchased from SIGMA-ALDRICH CO., GERMANY) in a concentration of 2% at different parts of body twice weekly for six months by mean of a gentle brushing.
- Group three (six rabbits) painted with PPD (2%) and administered antox (2 mg/animal/day) in the diet as antioxidant.

Experimental period lasts for six month, during this period the animals were observed for skin lesions and clinical signs. After the end of this experiment, all rabbits were slaughtered and samples from skin and internal organs were fixed in 10% neutral buffered formalin, prepared and stained with hematoxylin and eosin for histopathological examination. Blood samples were taken with anticoagulant for determination of total leucocytic count and differential leucocytic count.

Results

Six months after application, the hair coat of the rabbit became very dark, and the colored hair was very weak, falling down and easily detached when touched or handled. A large area of alopecia was appeared on the back of the animal at the site of application. Area of skin sensitization was appeared and the skin became hyperemic (red in color), painful and when handled the hair destructed and detached to leave area devoid from hairs besides necrosis of superficial keratinized layer (Fig. 1A, B). The group of rabbits treated with para-phenylenediamine and administrated antox showed similar clinical and gross features as appeared in the para-phenylenediamine treated group (Fig. 2).

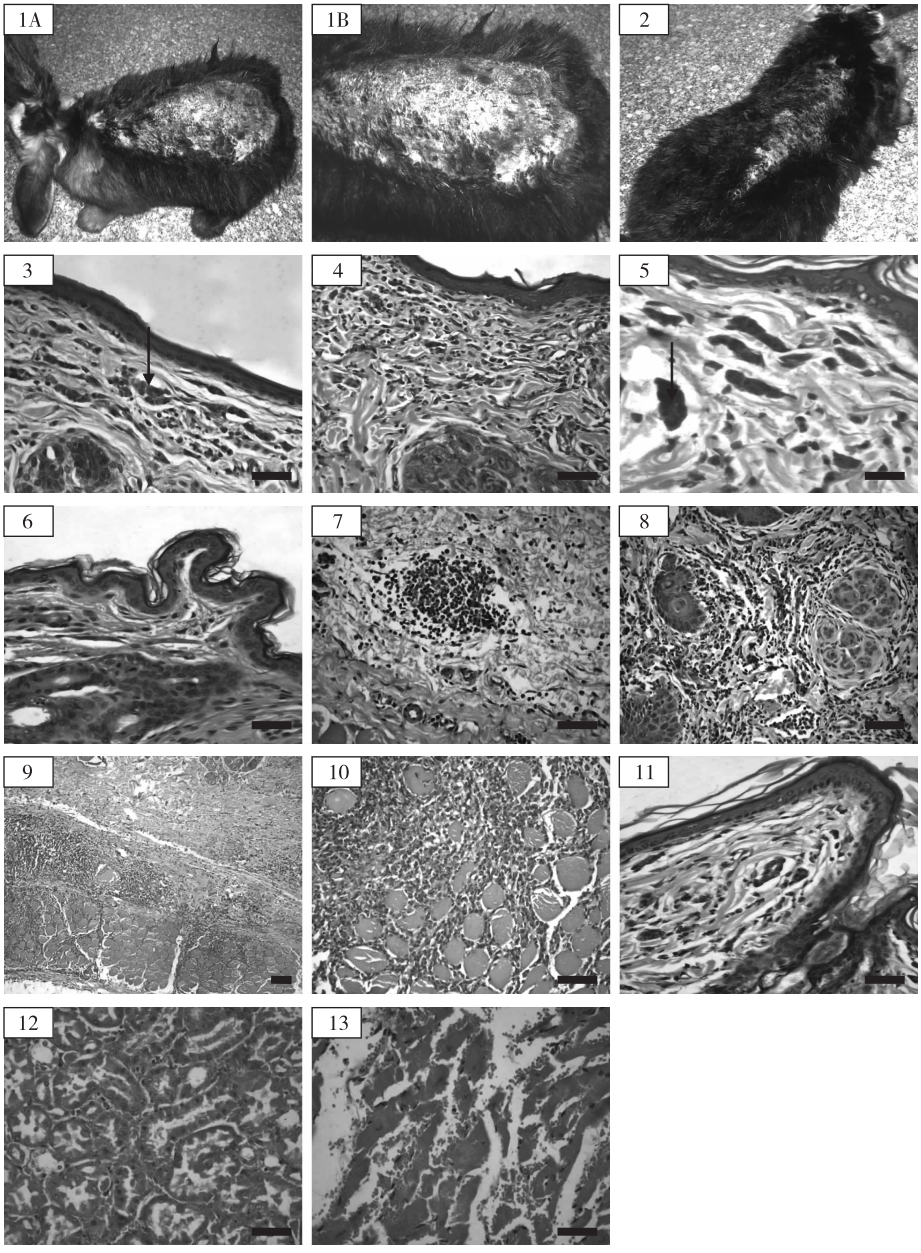

Fig. 1 A, B: Rabbit from PPD group showing black hairs with wide area of alopecia over the back of animal, redness and swelling of the area (signs of skin sensitization).

Fig. 2 Rabbit from PPD and antox group showing area of alopecia over back of animal, redness and swelling of the area.

Fig. 3 PPD group showing parakeratosis of keratin layer and cells bearing dye particles (arrow) with eosinophilic infiltration in dermis. H&E. bar = 50 μm.

Fig. 4 PPD group showing infiltration of eosinophil cells in dermis. H&E stain. bar = 50 μm.

Fig. 5 PPD group showing increased number of the cells contains particles of the dye (arrow). H&E stain. bar = 50 μm.

Fig. 6 Skin section from control group showing normal epidermis and keratin layer. bar = 50 μm.

Fig. 7 PPD group showing focal aggregation of eosinophils and oedema of the C. T. bar = 50 μm.

Fig. 8 PPD group showing folliculitis and perifolliculitis of compound hair follicles. bar = 50 μm.

Fig. 9,10 Showing heavy infiltration with eosinophil cells in the deeper layer of dermis and extend to the sub cutaneous muscle.

Fig. 11 Skin micrograph from PPD and antox group showing presence of cells bearing dye particles and eosinophil cells. H&E. bar = 50 μm.

Fig. 12 Kidney from PPD group showed necrobiotic changes in the epithelium of renal tubules and their lumen contain desquamated epithelium and hyaline casts. H&E stain. bar = 50 μm.

Fig. 13 Heart from PPD group showed hemorrhage with granular degeneration (arrow) and necrosis in the cardiac muscles. H&E. bar = 50 μm.

Histopathological results of examined skin sections showed that the dermis was seriously damaged while the epidermis showed only mild histopathological changes manifested by parakeratosis with no proliferative changes observed in the prickle cell layer with eosinophilic cell infiltration (Fig. 3, 4). Presence of macrophage cells containing particles of the dye present in the dermis (Fig. 5). When compared to skin section from normal control group (Fig. 6). The dermis was thickened and showed focal aggregation of intense inflammatory reaction consisted mainly of eosinophil cells and oedema in between C. T. (Fig. 7). Folliculitis and perifolliculitis of hair follicles which were encircled with some inflammatory cells and eosinophils. These cellular reactions extend deeply (Fig. 8, 9, 10). Similar changes occur in PPD plus antox group (Fig. 11). Lesions also reported in kidney and heart. A highly significant increase in the total leukocytic count was observed in para-phenylenediamine and para-phenylenediamine + antox groups when compared to control group. Prominent eosinophilia was observed in para-phenylenediamine treated groups.

Discussion

In the group of rabbits treated with para-phenylenediamine, the hair became black in color, very weak and fall down, leaving large area of alopecia. Necrosis of skin was also reported. Administration of antox did not inhibit the development of these lesions. Similar results obtained by [3]. Histopathological changes in the epidermis were represented by destruction of hair, defect keratinization and atrophy of keratinocytes. The diagnosis of allergic contact dermatitis in these group rabbits was also supported by the fact that the differential leucocytic counted revealed an intense eosinophilia in these rabbits. Increased number of the cells containing particles of the dye in the dermis in our opinion was due to absorption of the dye after application which then reached the circulation and causes lesions in the internal organs especially liver and kidney.

Conclusions

Application of para-phenylenediamine on the skin of rabbits was associated with allergic contact dermatitis in which the dermis was seriously damaged than the epidermis. Epidermal changes include destruction of the hair and folliculitis.

Administration of antox did not protect the skin of rabbits from the deleterious toxic effect of para-phenylenediamine.

The pathogenesis of the toxicity of para-phenylenediamine (PPD) for the internal organs was that para-phenylenediamine particles could be distributed from the skin and subcutaneous tissues within the macrophage cells via the circulation. Hence we declare that the process of tattooing is very serious and probably results in variable pathological affections of the internal parenchymatous organs including interstitial tubulonephritis, toxic hepatitis, myocarditis and rhabdomyolysis. So strict control of its practical uses should be adopted.

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Hygiene and Animal Health in Algeria

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Introduction

Algeria is a region that is characterized precisely by its tendency pastoral. Using different operating systems rangeland whose characteristics depend mainly on climatic conditions in this area, resulting in the dominance of arid and semi-arid areas. Sheep farming is one of the main resources of animal protein (MADR 2006). The sheep occupies an important place in the economy compared to other livestock animals (goat, cattle, camels and poultry).

To develop these animal productions, Algeria has developed an organizational structure for animal health services to improve the fight against infectious diseases.

Organisation for animal health

In Algeria the animal health is not a domain of activity integrated in the public health services. It doesn't exist, inside the governmental services, a formal structure that canalizes the ability and the competence of veterinarians to serve public health. The authorities themselves don't show any significant intention to integrate the service of the animal health in their global politics of public health.

The activities regarding the animal health consist mainly in fighting certain notifiable infectious diseases (foot and mouth disease, blue tongue poxvirus), the major zoonoses (rabies, brucellosis, and tuberculosis) and the inspection of meat and food of animal origin. These activities are led by the veterinary services of the Ministry of the agriculture (SPA 2005). Within each office of the agricultural services of district (Wilaya), there is a control veterinary service managed by a Veterinary Inspector. The latter has the following prerogatives:

– He has to take care of the application of laws and regulations concerning the living animals and food of animal origin inside and to borders of the country.

– To supervise the sanitary and medical prophylaxis.

– To help at the improvement of animal production by the vulgarization and the implementation of appropriate hygiene measures.

– To take care of the application of the regulation which are coming into force, concerning the living animals due to the exported, to be imported and those leaving the farm for the slaughter house.

– To inspect food animals with the purpose of detecting those suspected to be unfit to the human consumption.

– To ensure that adequate laboratory analyses.

With the internationalization of the economy during this XXI^{eme} century and the onset of diseases like B. S. E., the A. I. D. S, flu and the problem of the dioxin, the authorities became more receptive and reacted through the institution of new measures concerning human beings and animals.

The veterinary education is under the authority of the Ministry of Higher Education and Scientific Research. Before 1970, the education of veterinarians was achieved thanks to cooperation between Algeria and other states (France and Eastern European countries). In 1971, the first veterinary school was created to Algiers. Since this period, the path doesn't stop developing itself. Today six points of veterinary education within faculties can be found on the territory. Thanks to the creation of a new schedule, the number of veterinarians passed of 5 in 1970 to 10. 000 in 2011. (MESRS 2011)

The contents of programs dispensed within the different universities are uniform and have been inspired of the model of the French veterinary teaching, except for some matter (ex: diseases of pigs replaced by diseases of the camels). The teachers are post-graduated professionals or professors who have obtained of a specialization degree, abroad generally. Recently, the students can specialize in domains of animal sciences and animal health, in certain Algerian universities.

Demography increase in Algeria obliged the government to encourage animal production development with the help of grants from the banks and the provision of raw materials (cereals, oleaginous...) and of medicines.

The intensification of livestock farming led to some difficulties hardly controllable by the farmers, because the majority of them are illiterate (Bedouins) or don't

have the elementary educational bases for intensive livestock keeping. The creation of a "Hygiene office" in each township brought its contribution in the sanitary education of populations in field of the animal and also public health (information, projection of movies, display, exhibition, and technical aid)

The hygiene office helps the farmers in all operations of development in their herds.

Animal hygiene is part of the teaching curriculum of the future veterinarians. It is taught according to the type of livestock farming and to the animal species. It constitutes a very important stage in herd health appraisal and emphasis is put on prevention. (In accordance with the proverb: prevention is better than cure)

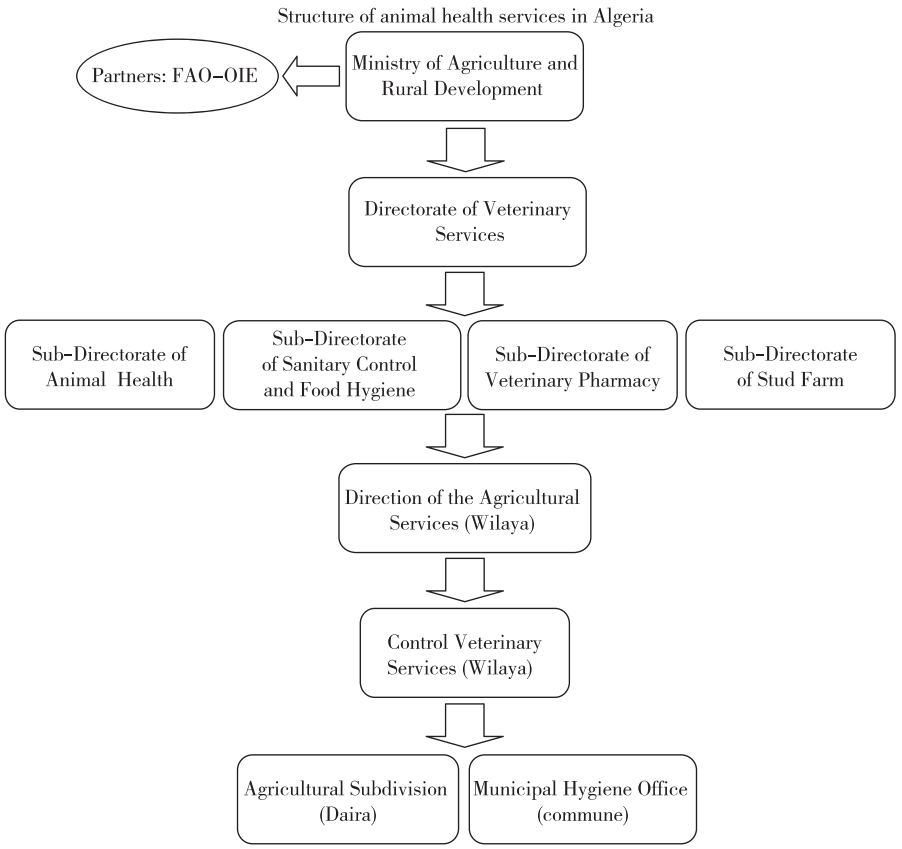
Hygiene doesn't only consist to the cleanliness and the decontamination of the premises. It especially concerns the sanitary and medical prevention. Practical measures related to hygiene are found in the routine management of the herds. Biotic and non biotic factors of the environment are concerned. To allow the animal to fully express its genetic potential, it is necessary to optimize its environment. Two categories of measure are necessary in animal hygiene:

Zootechnics measures: all non biotic factors, characterized by the physico-chemical parameters of the environment (temperature, humidity, radiations, air quality (noxious gas, dust...)...) and management conditions (density of population, buildings). Their variation can lead to the onset of various diseases and to stress. New scientific approaches like écopathology allow a global approach to health/disease. By the way the risk factors at the herd level can be assessed. The latter are then used as a base for disease prevention.

Biosecurity: relates to the measures supposed to avoid herd contamination. They especially aim the pathogens. Epidemiology is aimed at informing us about the health status of the farm animal region or nationwide. It also inform about disease spreading.

To me, finally Animal hygiene is strongly with veterinary public health in relation to animal production. Animal hygiene covers all these aspects of applied epidemiology, and beyond that it has to do with environmental protection in relation to animal keeping. The principales on which animal hygiene is established have their roots in sciences obviously tike animal sciences' but also ecology, ecophysiology, the bioclimatology, the ethology, ergonomics, to make it short animal hygiene stands at the confluent of several scientific disciplines.

This particular position might be found uncomfortable since there is the need to permanently gather knowledge from people involved in a more specialized scientific territory. In that, animal hygiene must not be confined to veterinarians but it has to be open



to other scientists whose field of interest falls within the scope of animal hygiene.

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Penicillin G and Oxacillin Resistance in *Staphylococcus aureus* Strains Isolated from Bovine Subclinical Mastitis

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Summary: Sixty-eigth out of 530 different *S. aureus* field strains isolated from subclinical cases of bovine mastitis from Germany (n=26), Indonesia (n=16), Mexico (n=16) and Brazil (n=9), respectively, were selected to be studied in the present work. The strains were tested phenotypically as well as genotypically for the presence of penicillin G- and oxacillin-resistance. For the primary phenotypical species identification of the 530 *S. aureus* strains, plasmacoagulase-test and Api 32_{Staph} system was applied. This was confirmed by molecular detection of the *S. aureus* specific genes encoding 23S rRNA, thermostable nuclease (*nuc*), clumping factor (*clf*A), coagulase (*coa*) and protein A region Xr (*spa*). The selection of the 68 strains was carried out by the random selection of one up to two strains per herd; additionally only strains with differences in size polymorphism of *coa* and Xr region of the *spa* gene and different macrorestriction profiles were included here. Genotypic resistance to semisynthetic penicillins (methicillin/oxacillin) and penicillin G was studied through the identification of *mec*A-and *bla*Z-genes, respectively. The *mec*A gene was detected in only one *S. aureus* isolate from Brazil which was not phenotypically resistant against semisynthetic penicillins as shown by the use of standard disc diffusion method, BBL-Chromoagar and MIC-determination by Vitek II. In contrast penicillin-resistance of strains based on the presence of the *bla*Z gene could be observed in 50 (73.5%) of the investigated strains. However, only 40 (58.8%) of these 50 *bla*Z-positive strains were phenotypically penicillin-resistant. According to the presented data resistance to semisynthetic penicillins in *S. aureus* field strains seems to be not a major problem in dairy herds of the investigated countries despite the long-term use of these antiinfective substances in the field.

Introduction

Staphylococcus aureus is one of the major pathogens responsible for a large variety of serious diseases in both human and veterinary medicine. One of the main problems caused by *S. aureus* which lead to great economic losses in the dairy industry is mastitis [1]. Recently, the emergence and the widespread of methicillin-resistant *Staphylococcus aureus* (MRSA) strains became a real threat for international public health. This spread of MRSA led to the lack of efficient alternative therapeutic drugs in hospitals [2]. Beside human cases, MRSA was isolated from several species of animals such as mares suffering from metritis [3, 4], wound infections [5-7], bovine mastitis [8] and also from diseased dogs, cats and rabbits [5, 9, 10]. Recently, MRSA of the clonal lineage ST398 and ST9 were identified from swine [11]. The transmission of MRSA between humans, animals and the environment was reported where animals live in close contact with human MRSA carriers. Concerning the role of MRSA in the induction of mastitis, MRSA was found to be responsible for sporadic cases of mastitis in humans [12] and from cases of subclinical bovine mastitis [8, 13]. However, the strains isolated from cows could not be distinguished from those isolated from the workers in close contact [8].

The possible transmission of MRSA from bovine subclinical mastitic milk to humans is a real concern due to widely use of cloxacillin for more than 30 years. This kind of semisynthetic penicillin is in use in many countries involved in the present study. For rapid and accurate diagnosis of MRSA, different molecular and cultural methods were developed [14-17].

Material and methods

In the present study 68 out of 530 *S. aureus* strains isolated from 4 different countries in 3 continents were investigated phenotypically and genotypically for resistance against oxacillin and penicillin G. These strains were isolated from cows suffering from subclinical mastitis in Germany, Indonesia, Mexico and Brazil. The selection of the strains was based on size polymorphism of *coa* gene and Xr region of *spa* gene and profile heterogenicity through the use of macrorestriction analysis with pulsed field gelelectrophoresis. Only strains with

different genetic profiles were further investigated.

The aim of the present work was to investigate the role of MRSA in the induction of bovine subclinical mastitis, and their impact in public health. Additionally the results of the present study give an update on the prevalence of penicillin G-resistant *S. aureus* isolates in bovine subclinical mastitis on the basis of genotypic strain selection.

Results

The 530 *S. aureus* isolates from mastitic milk samples, were identified by the use of the plasmacoagulase-test and the commercial identification system API 32_{Staph}. This was confirmed genotypically by PCR amplification of *S. aureus* specific gene segments encoding the 23S rRNA, thermostable nuclease (*nuc*), clumping factor (*clf*A) and coagulase (*coa*) and the gene segments encoding the *Xr* respective region and the IgG binding region of protein A (*spa*). Depending on the size polymorphism of gene *coa* and Xr-region of gene *spa* and according to macrorestriction profiles, 68 not related *S. aureus* isolates were further investigated for their antibiotic sensitivity. Fourty field strains were resistant to penicillin G determined by phenotypic and genotypic methods, ten isolates were genotypically *bla*Z-gene positive, but did not show any resistance by phenotypic testing. The genotypic investigation for the presence of gene *mecA* among the 68 investigated *S. aureus* cultures revealed the presence of the gene in a single isolate originating from Brazil. However, this field strain was according to the results achieved by the use of BBL-Chromoagar, standard disc diffusion methods and MIC determination sensitive towards oxacillin. In contrast to oxacillin-resistance and *mecA* gene, the genotypic and phenotypic examination of penicillin G-resistance and gene *blaZ* showed otherwise the spread of *blaZ* gene among the selected strains of 4 countries from 3 continents. In total, most of the investigated *S. aureus* isolates harboured *blaZ* gene (50, 73.5%). In detail, 18 (65.2%) german, 11 (68.75%) mexican, 8 (80.0%) indonesian and 13 (81.3%) brasilian field strains were positive for *bla*Z-gene by genotypic examination by PCR. In contrast only 40 (58.8%) isolates were penicillin G resistant phenotypically. The exact distribution was 10 (38.5%) field strains in Germany, 5 (13.3%) in Mexico, 5 (50%) in Indonesia and 10 (62.4%) in Brazil.

Discussion

The present data support other published data concerning the complete absence or the low incidence of MRSA in dairy samples. This indicates that MRSA has not yet emerged as a major pathogen in dairy herds despite long-term use of semisynthetic penicillins in this therapeutic area. The low incidence of detected MRSA strains in milk samples suggest the possible contamination of the milk samples by human or environmental strains.

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Impact of Biological Stress Factors in the Breeding Environment on Broilers' Immune Function

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Summary: To better understand the effect of different breeding environments on broilers' immune function, the concentrations of aerobic bacteria, fungi, and endotoxins in the air of the chickens' houses were determined periodically, and meanwhile, Newcastle Disease antibody(ND-Ab) levels and immune organ indexes of broilers were detected. In this study, 320 one-day-old healthy Arbor Acres(AA) broilers were randomly divided into two groups, i.e., Experimental Group(EG) and Control Group(CG), and bred in the chicken houses of different sanitary environmental management conditions. The experiment was done twice(Trial 1 and Trial 2). Determin concentrations of the airborne aerobic bacterial, the airborne fungal, the airborne endotoxin and detect the ND-Ab levels and thymus index. The results showed that, poor quality of a chicken house resulted in increase of microbial aerosol level, and negatively affected broiler body's immune function.

Key words: breeding environments; airborne microorganism; ND-Ab levels; immune organ indexes

Introduction

With the development of intensive and large-scale livestock & poultry production systems, animal health and welfare have attracted extensive attention. Air quality is an important index of the environmental quality, while microbial aerosols are the main indications of the environmental quality(Dutkiewicz et al., 1994; Zucker and Muller, 2000; Chang et al.,2001; Kaliste et al., 2002). Airborne microorganisms and their metabolites in a livestock & poultry house are main factors affecting animal health(Crowe et al., 1996; Urbain et al., 1996; Duan et al., 2008). And it is a key point for understanding the relationship between environment and animal's health to explore the impact of microbial aerosols on animal's immune function.

Material and methods

320 one-day-old health commercial-generation AA broilers were randomly assigned into two groups equally, and bred in two chicken houses. The two chicken houses were of completely same structure, but one is sterilized every two days and the other one is not cleaned.

Collect and culture the airborne aerobic bacteria, airborne fungi, and airborne endotoxins, then, measure ND-Ab levels and immune organ indexes. Data analysis was carried out with SPSS11.5 and Excel Statistical Software.

Results and discussion

In the two trials, the airborne aerobic concentrations of the EG were in the range of $(2.9-5.12)\times10^6$ CFU/m^3 air and $(2.76-10.99)\times10^6$ CFU/m^3 air, respectively, while those of the CG were in the range of $(0.57-1.83)\times10^6$ CFU/m^3 air and $(0.86-1.74)\times10^6$ CFU/m^3 air, respectively; the airborne aerobic concentrations of the EG were higher than those of the CG; furthermore, with increasing day age, the airborne aerobic concentrations of the EG were increasing, while those of the CG became stable.

In Trial 1, the airborne fungal concentrations of the EG and the CG were in the range of $(2.8-5.6)\times10^5$ CFU/m^3 air and $(0.72-1.1)\times10^5$ CFU/m^3 air ($P<0.05$). In Trial 2, the airborne fungal concentrations of the EG and the CG were in the range of $(0.27-1.21)\times10^5$ CFU/m^3 air and $(0.11-0.37)\times10^5$ CFU/m^3 air($P<0.05$). During the two trials, the fungal concentration profiles of the EG were gradually increasing, while those of the CG were relatively stable.

During days 15 – 45, in Trial 1, with increasing day age of the experimental broilers, the endotoxin concentration of the EG ranged from 2.5×10^3 to 16×10^3 EU/m^3 air rapidly, while that of the CG fluctuated between 2.4×10^3 and 3.1×10^3 EU/m^3 air($P>0.05$); in Trial 2, the endotoxin concentration of the EG had fluctuation between 0.8×10^3 and 17.07×10^3 EU/m^3 air rapidly, while that of the CG changed slowly in the range

of (0.4 – 2.13) × 10^3 EU/m^3 air ($P > 0.05$). The endotoxin concentration profiles of the two trials were very similar.

In Trial 1, the ND-Ab levels of the EG were gradually higher than those of the CG on days 14, 21, and 28; however, there was no significant difference between the two groups ($P > 0.05$). The ND-Ab levels of the two groups reached the maximum values, i.e. 6.1 ± 1.20 and 5.3 ± 0.67, respectively. However, the ND-Ab levels of the CG were significantly higher than those of the EG ($P < 0.05$) on days 35, 42, and 49; on day 49, the ND-Ab levels of the CG and the EG were 4.8 ± 1.03 and 3.6 ± 1.26, respectively. In Trial 2, on days 14 and 21, there was no significant difference ($P > 0.05$); however, on days 28, 35, and 42, there were significantly differences ($P < 0.05$); on day 49, there were very significantly differences. The antibody titers of the EG and the CG on days 28 and 49 were 5.5 ± 1.00 and 6.25 ± 1.29, and 3.8 ± 0.89 and 4.7 ± 0.92, respectively.

The results of Trial 1 showed that, on day 21, there were minimal differences of spleen indexes, BF indexes, and thymus indexes of the broilers between the two groups; on day 35, the thymus indexes of the EG and the CG were 2.31 ± 0.51 and 3.36 ± 0.72 ($P < 0.05$), respectively; on day 49, the spleen indexes of the EG and the CG were 1.06 ± 0.28 and 1.18 ± 0.14 ($P < 0.05$), respectively, while the thymus indexes of the EG and the CG were 1.79 ± 0.25 and 2.92 ± 0.73 ($P < 0.01$), respectively. There were significant differences in the spleen indexes and the thymus indexes between the two groups, but no significant difference in BF indexes ($P > 0.05$). The results of Trial 2 showed that, on the three sampling dates, the spleen indexes and the BF indexes of the CG were higher than those of the EG; however, there was no significant difference ($P > 0.05$). Nevertheless, the results through comparison of the thymus indexes between the two groups were similar to those of Trial 1.

Conclusions

The results of this study demonstrated that, due to foul breeding environment, a great amount of biological stress factors accumulated had unfavorable impact on immune organ development and ND-Ab levels. Therefore, it is a key point for healthy breeding to do well breeding sanitary management of animal houses and keep good immune resistance of animal body.

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Impact of Biological Stress Factors on Broiler Health and Production Performance

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Summary: 320 one-day-old healthy AA (Arbor Acres) broilers were divided randomly into the test/control groups to assess the impact of biological stress on production performance. The indexes of main biological stress (airborne concentrations of aerobic bacteria, fungi and endotoxin) and production performance (carcass traits, appearance and gait scores, and cecal lactobacillus) were measured and analyzed. The results showed that test group had higher airborne concentrations of aerobic bacteria and fungi than the control ($P < 0.05$), but no significant difference in endotoxin contents ($P > 0.05$). Compared with chickens in control group, average body weight of broilers in test group reduced by 16.5% – 19.6%, and significant decreases of cecal lactobacillus ($P < 0.05$) and whole net carcass rate ($P < 0.05$) were both observed. In conclusion, the environmental biological stress contributed greatly to the chick production performance.
Key words: biological stress; production performance; health; impact

Introduction

Air quality in animal houses is of importance for health and production performance of animals (Cargill & Hartung, 2001; Radon et al, 2002; Seedorf & Hartung, 2002). Lots of studies concerning animal health welfare showed that high concentrations of airborne pollutants could affect production efficiency and health of animals (Banhazi et al, 2008b).

Direct evidence is, however, short of confirming the above viewpoint (Chang et al, 2000; Dutkiewicz et al, 1994; Zucker et al, 2000; Kaliste et al, 2002). The study was therefore conducted to observe how microbes in the air affect broilers' health and production performance by modeling different raising environments.

Material and methods

320 one-day-old healthy AA broilers were randomly divided into two groups: the test group and the control group, 160 broilers for each group, each group with four replicates.

This experiment included the following steps: a) Quantification of concentrations of airborne bacteria, fungi (Brachmann et al, 1964) and endotoxin (Limulus Amebocyte Lysate, QLC2100 Bio Whittaker, Walkersvile, MD); b) Determination of broiler production performance markers; c) Determination of the concentration of cecal lactobacillus; e) Statistical analysis.

Results and discussion

During the whole growth cycle, concentrations of airborne aerobic bacterial, fungal and endotoxin in the test groups were increased unceasingly with day ages, but those of the control groups did not vary so much. There was no significant difference of airborne aerobic bacterial, fungal and endotoxin concentrations between the two groups in the earlier period (before 30 d) ($P > 0.05$), but there was significant difference in the later period of the growth cycle ($P < 0.01$).

The main factor was likely to be different season, because this carried out in autumn and spring. But the concentrations were the same variation trend. The differences of concentrations between the two groups was building construction and hygiene effects, as had been previously reported (Banhazi et al, 2008b). Through statistical analysis, it suggested that, the air quality of the control group was better than the test group. It is obvious that, high-level microbial aerosols in the environment of the poultry house may pose hazards to animal health, and thus influence seriously the welfare level of broilers.

The mean body weights of the chicken flocks of the test groups were lower than those of the control groups by 19.6% (trial 1) and 16.5% (trial 2) ($P < 0.05$); the whole net carcass rates of broilers in the environment of the control groups were both obviously increased ($P < 0.05$); the breast muscle rate of broilers in the environment of the control group was increased by

13.78% (trial 1) and the counterpart in the trial 2 was enhanced by 47.73% ($P < 0.05$). Although no significant difference of slaughter rates was found between the two groups in the two trials, the slaughter weights of the control groups were higher than those of the test groups by 22.93% (trial 1) ($P < 0.05$) and 36.83% (trial 2) ($P < 0.05$), respectively. The results suggested that, the environmental conditions may directly influence animal's production performance, and better environmental quality produces better economic benefits. Although no statistical data directly suggested correlation between airborne aerobic bacterial level and diseases, many studies demonstrated that, increased level of airborne aerosols may result in decreased animal immunity, slowed growth, and decreased production performance (Dutkiewicz et al,1994; Crowe et al,1996; Wolinsky,2006). Our results were consistent with the previous studies.

Our research showed that there was significant difference of airborne microbial concentrations between the test group and the control group since 30-day age of the test broilers ($P < 0.05$), while appearance and gait of broilers had significant difference between the two groups, suggesting that there was a certain correlation between chicken house's airborne microbial concentrations and broilers' gait status. The reason for the results was possible that, biological stress factors inhibit the function of body's immune tissues, and then produce adverse effects on neurohumor equilibrium and physiological metabolism, leading to broilers' poor general condition and decreased motility.

In the trial 1, the 49-d lactobacillus counts of the test group and the control group were significantly differently ($P < 0.05$). In the trial 2, the 35-d and 49-d lactobacillus counts of the test group and the control group were meaningfully and significantly differently ($P < 0.01$).

A well-balanced microbiota, including a high number of lactobacillus, in the caecum may be an important factor for a chicken's resistance to bacterial infections (Tuyttens et al,2008). Our results in the two repetitive tests also showed that the difference of cecal lactobacillus concentration in broilers between control group and test group was significant in 50 day-old ($P < 0.05$; $P < 0.01$). The higher concentration of lactobacillus observed in control group could thus indicate that these birds invest more in a stand-by immune system, allowing them to react more efficiently against pathogenic organisms.

Conclusion

Taken together, the environmental quality may directly influence animal health and production performance. In intensified poultry production, special attention should be paid to the environmental and ecological control of animal houses, with cleaning and hygiene being maintained, to create comfortable living conditions for animals, thus producing corresponding production benefits.

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Clinicopathological Studies on Naturally Occurring Sheep Pulmonary Adenomatosis

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Summary: Sheep pulmonary adenomatosis (SPA) is a naturally occurring pulmonary neoplasm of sheep. After the first outbreak of it in 1985 in Inner Mongolia of China, 2 sheep were diagnosed again in 2006. The clinical signs, histological and ultrastructural pathology of two naturally occurring cases (Poll Dorset sheep, 3-years old) were investigated by regular pathological method, X-ray and transmission electron microscope in this research. Simultaneously, the dynamic changes of lymphocytes were examined in their peripheral blood by flow cytometry. The results showed that two natural cases produced watery nasal fluid, higher breath rate than healthy sheep and cloudy-shaped firm lesions in the lungs by X-ray. Histologically, the secretory epithelial cells in the pulmonary alveoli and epithelial cells of the bronchioli proliferated and formed papillar form that project into the alveoli or bronchioli. The affected pulmonary alveoli epithelial cells were replaced by the large numbers of tumorous cuboidal epithelial cells by transmission electron microscope. The number of $CD4^+$ T lymphocytes of peripheral blood presented a transitorily increase and rapidly decrease subsequently. These results will facilitate the diagnosis and quarantine of SPA, providing an opportunity for future epidemiological research.
Key words: sheep pulmonary adenomatosis; naturally occurring case; pathology; lymphocytes

Introduction

Sheep pulmonary adenomatosis (SPA), also known as ovine pulmonary adenocarcinoma and jaagsiekte, is a naturally occurring pulmonary neoplasm of sheep caused by jaagsiekte sheep retrovirus (JSRV) (DeMartini and York, 1997; Sharp and DeMartini, 2003). SPA was first emerged in South Africa in 1825. And then it occurs in many regions of the world including Inner Mongolia Autonomous Region of China and its incidence is difficult to evaluate in the absence of an appropriate screening tool, where prevalence in affected flocks can be as high as 10%. However, the differences in apparent rates of occurrence of SPA among continents and breed of sheep may reflect variations in viral strains and host susceptibility (Caporale et al., 2005). SPA represents a unique model of lung neoplasia and studies on its etiopathogenesis can yield further insights into the mechanisms of lung and epithelial neoplasms (Hecht et al., 1996; Palmarini and Sharp, 1997). The recent advances in understanding JSRV and the pathogenesis of SPA should lead to novel strategies for diagnosis and control of this disease and for its exploitation as a comparative model for human lung cancer, for which samples representing early stages of tumor growth are difficult to obtain (Griffiths et al., 2010; Martineau et al., 2011). The aims of this study were to investigate the pathological investigation on the naturally occurring cases of SPA and provide a reference for diagnosis of SPA.

Material and methods

Clinical signs examination: Studies were conducted with two SPA naturally occurring Poll Dorset sheep (3-years old). Six sheep from SPA-free flock at the suburb of Hohhot in Inner Mongolia were used as unchallenged controls (case Nos. 3 – 10). Two naturally occurring cases were observed and checked until death for clinical signs of respiratory disease and some parameters that might be suggestive of SPA, including the breathing rate.

X-ray examination: All the naturally occurring sheep and control sheep examined by X-ray of thorax at intervals to observe and compare the visible changes of lung.

Isolation of lymphocytes: The dynamic changes of lymphocytes were examined in peripheral blood of naturally occurring cases and control sheep by flow cytometry at the different stages. Peripheral blood samples were collected into EDTA tubes immediately at intervals until necropsy. Peripheral blood leukocytes (PBLs) were obtained by centrifugation after lysis of erythrocytes. Mouse anti-sheep monoclonal antibody (Serotec Co., Beijing) CD4 was used to isolate $CD4^+$ cells. Antibodies were used at the optimal dilution according to the manufacturer's specification.

Pathological investigation: The two naturally occurring cases and control sheep were dead naturally and

sacrificed humanely after 2 months. Macroscopic and histological examination of the tissues from naturally occurring cases were performed to identify any gross histopathological changes. And the tissue samples and the neoplastic-like areas of lungs were taken and placed in 10% buffered formalin and 30% glutaraldehyde for subsequent histopathological and ultrastructural examination, respectively, according to routine procedures.

Ethics: The study was approved by the Institutional Animal Care and Use Committee of China Agricultural University, and conducted in accordance with the bylaws of the committee.

Statistical methods: All data are presented as mean standard deviation. The naturally occurring cases were compared with the control sheep using parametric t tests by SPSS Statistics 17.0 software. Significant differences in the scores were established at P-values less than 0.05 ($P < 0.05$).

Results

Clinical signs: Raising the hind quarters and lowering the head of the naturally occurring sheep may cause frothy mucoid fluid to run from the nostrils. The breathing rate of them was significantly higher than the control sheep following the development of disease.

X-ray examination: All of the naturally occurring sheep were presented the cloudy-shaped firm lesions in the lungs by X-ray examination in adaxial-abaxial view of the thorax (Fig. 1). And the bronchiectasis and trachea thickness were occurred in lateral view of the thorax. The diffused cloudy-shaped firm lesions in the lung and confined to the diaphragm lobar increased gradually. The white dots regions in the lung were suggested the tumor nodules in imaging.

Immunophenotypic profiles: To investigate possible phenotypic anomalies arising from JSRV infection, lymphocytes isolated from peripheral blood were stained with appropriate monoclonal antibodies used for subset purification. The main characteristic was a mean reduction in the levels of $CD4^+$ T lymphocytes in PBLs (percentage number of $CD4^+$ cells were ranged for 22.040 ± 2.731 to 16.640 ± 2.132 in per ten thousand cells) compared to the normal values from unaffected sheep (range, 28.870 ± 1.215 to 33.185 ± 1.860) (Table 1).

Table 1 The $CD4^+$ T lymphocytes of naturally occurring SPA cases in blood

Testing times	Control sheep	SPA sheep
1	28.870 ± 1.215	$16.640 \pm 2.132^{**}$
2	31.930 ± 2.085	$23.675 \pm 1.043^{*}$
3	36.318 ± 1.293	$25.415 \pm 1.103^{*}$
4	33.878 ± 0.628	$14.305 \pm 1.785^{**}$
5	36.830 ± 2.140	$23.110 \pm 2.112^{**}$
6	33.185 ± 1.860	$22.040 \pm 2.731^{*}$

* To show the data of control and SPA sheep had evident difference ($P < 0.05$);

** To show the difference between data of control and SPA sheep is very significant ($P < 0.01$).

Necropsy: In naturally occurring sheep, gross lesions are confined to the lungs and the lungs were considerably enlarged (Fig. 2) and its surface and cut surface showed numerous firm, differently-sized and pinkish-gray nodules. The lesions usually consisted of a gray coalescing mass that effaced the ventral portions of the cranial or middle lobes of one or both lungs. The cut surface of the tumor masses often exuded clear to slightly opaque fluid. White frothy fluid may be seen in the bronchi and bronchiole at necropsy; Some cases were found that chronic pleurisy with fibrous adhesion which made it difficult to remove the lungs from the thorax.

Histopathology: The lung sections revealed the

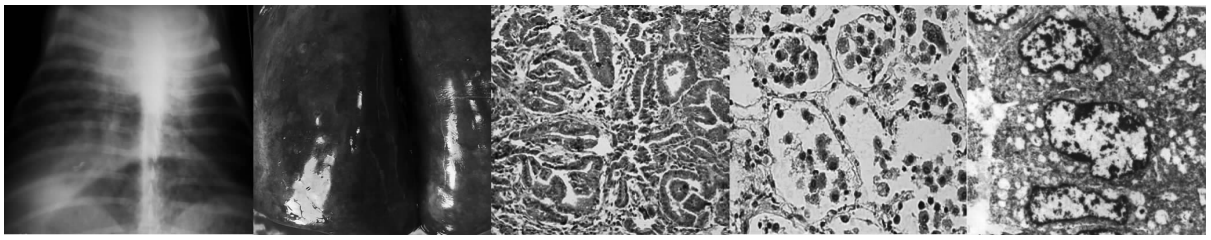

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Fig. 1 The naturally occurring SPA sheep was presented the cloudy-shaped firm lesions in the lungs by X-ray examination in adaxial-abaxial view of the thorax during the disease; Fig. 2 In SPA sheep, gross lesions are confined to the lungs and the lungs were considerably enlarged and its surface showed numerous firm, differently-sized and pinkish-gray nodules; Fig. 3 The epithelial cells neoplastic proliferated and formed papilliform growths that project into the alveolar or bronchioli in naturally occurring SPA sheep, HE staining; Fig. 4 The large numbers of macrophages accumulated in the alveoli adjacent to the neoplastic lesions in naturally occurring SPA sheep, HE staining; Fig. 5 Electron micrographs, the neoplastic secretory epithelial cells in the pulmonary alveoli of naturally occurring SPA sheep and the bronchioli located at the fundus and arranged in monolayer

presence of several foci of epithelial cell neoplastic proliferation in alveolar regions (Fig. 3). The epithelial cells neoplastic proliferated and formed papilliform growths that project into the alveolar or bronchioli, and large numbers of macrophages accumulated in the alveoli adjacent to the neoplastic lesions (Fig. 4). And this result also has been confirmed in the previous researches (Jassim et al., 1987).

Transmission Electron Microscopy: The nucleus of secretory epithelial cells in the pulmonary alveoli and the bronchioli located at the fundus and were round in shape, had been seen more different-sized secretive bubbles in the cytoplasm in the pulmonary tissues (Fig. 5). The affected pulmonary alveoli epithelial cells were replaced by the large numbers of tumorous cuboidal epithelial cells and some neoplastic cuboidal cells contained denaturalized lamellar bodies in cytoplasm by transmission electron micrograph in naturally occurring cases.

Discussion

Epidemiology investigation indicated that the coughing and inappetence are not common in naturally occurring cases but, once clinical signs are evident, weight loss is progressive and the disease is terminal within weeks or months. Most affected sheep produce 100 – 200 ml/day of watery nasal fluid that originates in the lungs ("wheel barrow test" is positive) and it is an important criteria for the diagnosis of SPA (Hecht et al., 1996).

The X-ray examination indicated that the tumor nodules were more obvious along with development of the disease and the severity of the signs reflects the extent of tumor development in the lungs. And the trachea thickness reflects the high frequency respiratory movements of affected sheep. Thereby, the method of X-ray has the diagnosis meaning for SPA affected sheep in living.

The main characteristic of phenotypic anomalies arising from JSRV infection was a mean reduction in the levels of $CD4^+$ T lymphocytes in PBLs, confirming a previous report (Rosadio and Sharp, 1992). SPA-affected sheep lack circulating anti-JSRV antibodies and have an increased susceptibility to secondary bacterial infection (Verwoerd, 1990). This observation could indicate that the immune system of affected sheep had been damaged in a certain extent.

Ultrastructural findings indicated that the tumor cells exhibited variable degrees of differentiation. Our data indicate that SPA tumor nodules may be primarily composed of AT II cells (Oda et al., 2011). These results indicated that JSRV primarily transforms AT II cells, although the tumors are frequently heterogeneous.

Conclusions

In summary, the present study conducted the pathological investigation on the naturally occurring cases of SPA. The results of this work can be a reference for diagnosis of SPA and it will be helpful for quarantine of SPA sheep, providing an opportunity for future epidemiological research. Furthermore, future investigations in relation to fast diagnosis methods of SPA are necessary.

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Does the Protein Source Affect Litter Quality and Health of Foot Pads in Fattening Turkeys?

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Summary: Nutrition is considered to be a major factor in the onset of foot pad dermatitis (FPD) along with poor litter quality. A high dietary level of soybean meal (SBM) is thought to be one of the nutritional factors causing FPD. The aim of this study was to evaluate the effects of different diet formulations, which varied in terms of protein ingredients (i.e: plant or animal) on development and severity of FPD in fattening turkeys. Two consecutive trials were done at the university research farm with using wood shavings and straw as a bedding material. In each trial, the birds were divided into 2 groups. One group fed a "plant protein diet", based on wheat, yellow corn, SBM and rape meal. The second group was fed an "animal protein diet", based on wheat, yellow corn, SBM (low levels), fish meal and haemoglobin powder. At the end of fattening period (16 wk) in each trial, the foot pads were externally assessed (600 foot pads in first trial for both groups/573 foot pads in second trial for both groups). Excreta and litter samples were collected weekly (only in second trial) to determine development of moisture. FPD severity was significantly higher for birds fed all-vegetable diets compared with those fed animal protein diet in both trials. Feeding birds animal protein diet resulted in reduction in foot pad lesions by ~ 40% and ~ 25% for trials (1/2), respectively. Moreover, the moisture contents of excreta and litter from birds fed a diet with the inclusion of plant protein diet were significantly higher to that obtained with animal protein diet. The protein source seems to have a significant effect on the development of FPD mediated either by its content of potassium or galactosides (stachyose and raffinose).

Introduction

Foot pad dermatitis (FPD) is a widespread challenge in turkey production. The prevalence of FPD in turkeys is extremely high [4]. About 97.2% of turkeys showed FPD lesions with no marked effects on the body weight of five different strains of male turkeys at the end of the fattening period [5]. Nutrition is considered to be a major factor in the onset of FPD along with poor litter quality. A high dietary level of soybean meal (SBM) is thought to be one of the nutritional factors causing FPD. It is well known that SBM is the most common protein source for use in turkey diets. JENSEN et al. [6] reported that poults fed diets with increased amounts of SBM produced feces that apparently increased the incidence of FPD. The nonstarch polysaccharide (NSP) fraction of the SBM (especially stachyose and raffinose) has poor digestibility that results in sticky and potentially irritant droppings and wet litter [6]. It also assumed that higher levels of potassium (K) in diets of high SBM lead to a higher moisture content in the litter as a result of increased water intake [3].

According to European Union regulations, animals grown for human consumption cannot be fed animal by-products. The aim of this study was to evaluate the effects of different diet formulations, which varied in terms of protein ingredients, on the development and severity of fattening turkeys.

Material and methods

Two trials were done at the university research farm. A total of 5096 (trial 1) and 5112 (trial 2) female BUT Big 6 turkeys took part in the investigation. All were housed under identical husbandry conditions and going through a normal fattening procedure. In first trial, the birds were divided into 2 groups fed either plant protein diet or animal protein diet for about 112 days old (11.11.2011 till 02.03.2012). Also in second trial, the birds were divided into 2 groups fed either plant protein diet or animal protein diet for about 110 days old (14.03.2012 till 02.07.2012). In both trials, each group being allocated to a floor pen (472 m^2). Each pen was littered with wood shavings to a depth of approximately 4 – 6 cm over the floor (7.60 kg/m^2). Feed and water were available *ad libitum* for all groups. Only in second trial, the dry matter (DM) contents of litter and excreta were measured. Litter samples for measuring the moisture were collected weekly from 6 sites (4 peripheral and 2 central sample), then thoroughly mixed. A subsample of about 200 g was taken to assess the DM content. Samples were oven-dried at 103℃ for the time needed to reach constant mass. Two pooled pure fresh excreta (70 g) samples were collected individually from the surface of

litter in each group for determination of the DM content weekly. At the end of fattening period in each trial, the foot pads were assessed (600 foot pads in first trial/573 foot pads in second trial). Only the central plantar area was scored, signs of foot pad lesions were assessed on a 7-point scale (0 = normal skin; 7 = over half of foot pad is covered with necrotic scales) according to MAYNE et al. [8].

Results

Table 1 shows the body weights of fattening turkeys which were recorded every 2 weeks throughout the experimental period. In first trial, no significant differences were noted throughout the fattening period between birds fed plant protein or animal protein diets (except at: 2, 12, 14 weeks of life). At end of the fattening period (16 week old), no significant differences were observed. Nevertheless, in second trial at 16 week of life birds fed plant protein diets were significantly higher (10383 g) compared with those fed animal protein diets (10075 g). Furthermore, in both trials birds fed animal protein diets had a favourable feed conversion ratio (FCR) in comparison to those fed only plant protein diets (2.55 vs. 2.63, respectively in first trial; 2.39 vs. 2.75 respectively in second trial).

Table 1 Comparison of turkey's body weight (g) at different times (Mean ± SD)

	Weeks of life							
	2 (n = 60)	**4 (n = 60)**	**6 (n = 60)**	**8 (n = 60)**	**10 (n = 60)**	**12 (n = 60)**	**14 (n = 60)**	**16 (n = 60)**
First trial (diet)								
Plant protein	$335^{a} \pm 33.1$	$1201^{a} \pm 114$	$2327^{a} \pm 228.3$	$3855^{a} \pm 394.5$	$5718^{a} \pm 523$	$7808^{a} \pm 612$	$9938^{a} \pm 582$	$11392^{a} \pm 772$
Animal protein	$322^{b} \pm 29.9$	$1184^{a} \pm 128.4$	$2334^{a} \pm 291$	$3906^{a} \pm 359$	$5596^{a} \pm 486$	$7550^{b} \pm 782$	$9545^{b} \pm 686$	$11309^{a} \pm 769$
Second trial (diet)								
Plant protein	$332^{a} \pm 32.8$	$1013^{a} \pm 90.4$	$1972^{b} \pm 191$	$3582^{a} \pm 374$	$5436^{a} \pm 463$	$7549^{a} \pm 627$	$9407^{a} \pm 656$	$10383^{a} \pm 605$
Animal protein	$304^{b} \pm 40.1$	$953^{b} \pm 100$	$2084^{a} \pm 221$	$3677^{a} \pm 401$	$5447^{a} \pm 336$	$7493^{a} \pm 592$	$9098^{a} \pm 581$	$10075^{b} \pm 576$

[a,b] Means in the same column in each trial with different superscripts are significantly different ($P < 0.05$).

The mean of the different moisture contents of excreta and litter are shown in Table 2. It was observed that birds fed animal protein diets were associated with significantly higher DM content of excreta (21.9%) compared with those fed plant protein diets (20.5%).

Table 2 DM contents (%) of the litter and excreta only in second trial and NH_3 (ppm) in the air (3 cm above floor) of both trials (1/2) during the experiment (Mean ± SD)

	Plant protein diet	**Animal protein diet**
Excreta DM, % (n = 28)	$20.5^{b} \pm 1.87$	$21.9^{a} \pm 2.59$
Litter DM, % (n = 84)	$72.3^{b} \pm 12.5$	$76.5^{a} \pm 9.83$
NH_3 (ppm) (n = 8/8)	$11.0^{a} \pm 14.8$/ $4.63^{a} \pm 3.62$	$14.0^{a} \pm 19.3$/ $3.63^{b} \pm 3.11$

[a,b] Means in the same raw with different superscripts are significantly different ($P < 0.05$).

Furthermore, DM content of litter was significantly affected by the type of diet (Table 2). It was observed that birds fed animal protein diets had significantly higher DM content of litter (76.5%) in comparison to those fed plant protein diets (72.3%).

Mean external scores of foot pads at end of fattening period of turkeys in both trials are presented in Table 3. In first trial, it was noted that birds fed animal protein diets had significantly lower FPD scores (3.50 ± 0.887) vs. (5.85 ± 1.06) for birds fed plant protein diets. Similarly, in second trial significant differences were observed between birds fed animal or plant protein diets (3.59 ± 0.915 vs. 4.65 ± 0.696, respectively).

Table 3 External foot pad scores of turkeys at end of fattening period (Mean ± SD)

Trial	**Plant protein diet (n = 300/287 foot pads)**	**Animal protein diet (n = 300/286 foot pads)**
1	$5.85^{a} \pm 1.06$	$3.50^{b} \pm 0.887$
2	$4.65^{a} \pm 0.696$	$3.59^{b} \pm 0.915$
Prevalence of FPD scores (%, trials 1/2)		
Low = 0 – 3	0.350/1.65	40.0/39.6
Medium = 4 – 5	39.75/88.9	59.35/58.7
High = 6 – 7	59.9/9.45	0.650/1.70

[a,b] Means in the same column with different superscripts are significantly different ($P < 0.05$).

Discussion

Dietary changes leading to higher water intake by birds are expected to increase excreta and litter moisture. Many factors impact water intake in birds, but for feed formulations based on common ingredients, electrolytes play a major role [3, 11, 1]. Diets with increased Na and K result in an increased water intake and litter moisture [1]. Soybean meal contains several constituents which could be responsible for a higher water intake and excretion such as K. This principle supports the litter moisture responses obtained in this study. Therefore, the increased moisture in litter from birds fed the plant protein diets when compared with the animal protein diets

may reflect an increase in water intake due to higher contents of Na and K levels in these diets. Moreover, the indigestible oligosaccharides in SBM have been implicated in causing sticky excreta and wet litter problems [9]. Also, BEDFORD [2] stated that SBM contains α-galactosides (raffinose and stachyose) which cause the excreta to have hydroscopic properities and hence resulted in wet litter conditions.

The key point is that the prevalence and severity of FPD were clearly affected by the litter quality. Improving the general standards of rearing, considering feeding, housing facilities, equipment, management and stockman ship should be considered as these factors are mainly related to the animals' welfare. Litter moisture is considered to be a leading factor predisposing to FPD [8, 7]. Even short exposure to wet litter around feeding or drinking places may result in a markedly increased prevalence and severity of FPD. A significant higher FPD scores in the current study for birds fed plant protein diets than those fed animal protein diets are positively correlated with the DM contents of both excreta and litter. It is clear from the results that the major factor leading to FPD is the litter moisture. Also, in this study the inclusion of high levels of SBM in the diet increased the severity of FPD. Similarly, it was found that the incidence of FPD was increased in birds fed diets with high levels of SBM [6, 9]. Moreover, NAGARAJ et al. [9] concluded that no effects of protein levels on foot pad lesions in broilers. Therefore in this study, the effect of high dietary levels of SBM on FPD could be related to its content of both K (producing watery excreta) and oligosaccharides (producing sticky excreta). VIEIRA and LIMA [10] observed increased water intake and higher moisture in the excreta when broilers were fed formulated exclusively with plant ingredients vs. a diet with up to 8% animal by-products with less K.

Conclusions

Out of this observation, it can be concluded that the control of litter moisture (through proper feed formulation) is likely to be highly effective in reducing the severity of FPD in commercial turkey flocks significantly. Birds fed all-vegetable diets based exclusively on corn and SBM have an increased potential to develop FPD as well as to produce excreta with higher moisture contents when compared with those fed diets with inclusions of fish meal haemoglobin powder. Moreover, from animal welfare point of view it is recommended not to fed turkeys diets based only on all-vegetable diets to reduce the percent of ulcers in foot pads.

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Conventional Detection of *Babesia bovis* in Tick Infested Cattle and Its Effect on the Hematological Profile

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Summary: The present study was conducted on thirty-seven cattle; five of them are considered control group. clinical examination of the animals concluded that there were different degrees of tick infestation and some common clinical signs recorded on the animals, such as fever ≥40℃, hemoglobin urea, paleness of the visible mucous membranes and various degrees of respiratory signs. The results of the current study revealed that the conventional method of diagnosis (blood films) was still recommended for day to day examination in clinically infected cases, especially in acute stage which gave infection rate (18.75%). Variable degrees of anemia ranged from severe to mild anemia according to the infection rate with significant decrease in the total RBCs, Hemoglobin %, MCT, MCV and MCH.
Key words: *Babesia bovis*, conventional diagnosis, anemia

Introduction

Babesiosis was first reported in 1888 by Viktor Babes in Romania who detected the presence of round, intra-erythrocytic bodies in the blood of infected cattle [3]. Bovine babesiosis is one of the TBD found worldwide which caused by different species of the genus Babesia [1]. In Egypt, first description was in 1947 [20]. *Boophilus* (*Rhipicephalus*) *annulatu* sticks were responsible for transmitting the disease. Parasites of the genus Babesia infect a wide variety of domestic and wild mammals as well as man [22]. The most species affecting bovine were studied are *Babesia bovis*, *B. bigemina* and *B. divergens* [4]. Bovine babesiosis had a huge economic impact due to either loss of beef production of infected animals or death of most of them. The clinical signs of *B. bovis* infection are fever, hemoglobinuria, acute anemia, and cerebral or nervous signs [13, 16, and 19], Animals that survive with *B. bovis* infection generally become low-level carriers of the parasite and serve as a reservoir for transmission [15]. The diagnosis of bovine babesiosis is an important tool to prevent and control of the dissemination of the disease. The routine clinical diagnosis for babesiosis is usually based on the microscopic detection of parasites from collected stained blood smears [3]. The costs of different methods of diagnosis must be added to the high costs of prevention, treatment, control of the disease and tick control program [23].

Material and methods

Animals

A total number of 37 cattle belong to different localities in Assuit, governorate were subjected to this study. 5 clinically healthy animals serve as control group and 32 animals were infested with tick with different degrees.

Sampling

Two blood samples were collected from each animal.

1. Blood sample was collected directly from the ear vein used for preparation of blood films.

2. Whole blood sample from jugular vein on EDTA vacationer tubes for Hematological analysis [6].

Methods

Clinical examination:

All animals in this study were subjected to clinical examination according to [25]. Some of those animals showed various degrees of the characteristic clinical signs for the babesiosis like fever (> 40℃), Hemoglobin urea, enlargement of the superficial lymph nodes (acute form), in appetence, paleness of the visible mucous membranes, various degrees of respiratory dyspnea. In addition to various degrees of ticks infestation.

Conventional diagnosis:

Thin blood films were prepared immediately after taking the whole blood samples direct from the ear vein in the field, allow these smears to dry by air then fixed by using methyl alcohol (methanol) for about 3 – 5 min, allow them to dry by air after fixation step then stained with Giemsa stain diluted at 8% with bidistalied water for about 30 – 45 min. Dried by air and examined on Olympus microscope by using Oil immersion lens at × 1000 magnification [5 and 6].

Hematological analysis:

Hematological analysis was carried out using automatic blood cells counter (Medonic CA 620, Sweden). The following parameters were measured; total red blood cells count (T. RBCs), haemoglobin concentration (HGB), mean corpuscular volume

(MCV), red blood cell distribution width (RDW), (RDWa), haematocrit (HCT), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC).

Statistical analysis:

Statistical analysis was conducted using SPSS 16.0 for windows (SPSS, Chicago, USA) and was carried out using one way analysis of variance. Data were expressed as mean ± SD.

Results and discussion

Babesiosis considered as a pasture infection and linked to vector ticks. In the current study thirty-seven animals were examined. Five animals were healthy (free from ticks, showed no clinical signs and free blood films) were used as control group. Thirty-two animals were infested with various degrees of tick infestation all of them investigated for the confirmation of bovine babesiosis (*B. bovis*) by using Giemsa stained blood films besides clinical signs of the disease (Fever, Hemoglobin urea). The infection rate was conventionally confirmed in six animals (18.75%) by detection of the intraerythrocytic stage. This finding came in agreement with [9]. It is not expensive but require an experienced microscopist to differentiate different species and is reliable only if the amount of parasites in the blood is high enough to be detected, which is usually possible during acute cases; this in agreement with [18 and 24]. Special attention must be paid to the source of the blood; peripheral blood is useful only for species like *B. bigemina*, *B. divergens*, or *B. gibsoni*, which do not adhere to the vascular endothelium. Some species like *B. bovis* or *B. canis* adhere to endothelial cells [2 and 14]. In Egypt, there are large numbers of cattle infested with subclinical babesiosis[11]. As infection cannot easily be diagnosed by examination of stained blood film, also, negative microscopic examination does not exclude the possibility of infection [26]. The results of complete blood cell count (CBC) showed regenerative anaemia. All collected blood samples revealed increase of MCV, MCH values and slightly decreased of MCHC value indicating persistence of macrocytic hypochromic anemia. The result of blood parameters of total erythrocyte count, HCT and hemoglobin showed significant decrease. Also there were significant decreases in total RBCS counts ($P < 0.01$), haemoglobin (Hb) concentration ($P < 0.01$), packed cell volume (PCV) ($P < 0.01$) in *Babesia bovis* infected group if it compared with the negative and control groups as shown in Table 1. The results of CBC were in accordance with the findings of [7, 8, 9, 10, 12, 17, and 21] who reported macrocytic normochromic anemia with acanthocytosis and spherocytosis, and also recorded significant decrease in total leukocytic count, red blood cell count, hemoglobin concentration and packed cell volume.

Table 1 Showing the Hematological findings in different groups

Animals' groups	RBC $\times 10^6/mm^3$	HGB(g/dl)	HCT(%)	MCV (μm^3)	MCH(pg)	MCHC(g/dl)	RDW(%)	RDWa(μm^3)
Control group	9.9 ± 0.064	10.88 ± 0.622	32.92 ± 2.612	33.20 ± 2.59	10.98 ± 0.634	33.10 ± 1.239	26.04 ± 1.905	23.90 ± 3.082
Negative group	6.91 ± 1.370**	10.15 ± 1.608	31.22 ± 5.872	45.39 ± 3.501**	14.88 ± 1.703**	32.75 ± 1.950	23.469 ± 2.121	33.246 ± 2.973
Positive group	2.57 ± 0.997**	3.866 ± 1.29**	12.15 ± 3.917**	48.017 ± 4.803**	15.38 ± 1.162**	32.117 ± 2.114	28.567 ± 9.44	40.450 ± 1.203

** P (<0.01) highly significant.

Conclusion

It could be concluded that tick infestation and Babesia infection in cattle are destructive obstacles for the livestock resulted in severe anaemia and cause huge economic losses in the production and reproduction of those animals. So tick control programs must be applied strictly with periodical examination of the animals by using Giemsa-stained blood films also if it is possible apply the molecular techniques to detect the chronic cases and carriers animal. It is recommended to supply supportive treatment to animals which suffered from babesiosis to help itto resume their normal productivity.

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Dynamic Ultrastructural Changes of Scolex on Cysticercus Pisiformis

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Summary: In order to research the ultra-morphological changes of scolex of cysticercus pisiformis during the relative rest and motion states, the ultrastructure changes of scolex located in cyst and evaginated from cyst after cultivation were comparatively observed by scanning electron microscope. The results showed that, when the scolex was in the relative rest states, from the top observed, the rostellum with the tegument muscular column that connected to tooth-hook looked like the umbrella and covered on the front end of the scolex. Viewed from the side of the scolex, the tooth-hook on the rostellum looked like the antler branch and had only one row. Four suckers looked like cavities were located in the back of the rostellum and distributed around the scolex in the equidistance. When the scolex was in the motion states, the tegument muscular column on the rostellum contracted, the antler-like tooth-hook extended to periphery, and the sucker also made the ring-like and longitudinal-like contraction. In brief, ultrastructure of the scolex of cysticercus pisiformis had apparent changes during relative rest and motion states. Those changes were able to help the invasiveness of the scolex to the host tissue.

Key words: cysticercus pisiformis, scolex, rostellum, sucker, ultrastructure

Introduction

Cysticercosis pisiformis in rabbit is one of proscolex diseases caused by cestode pisiformis and a worldwide disease [1-3]. It does the great harm to rabbit keeping. At present this disease is still serious prevalent in China [4-8]. It has been reported that the morbidity of this disease in some small and medium rabbitries is from 20% to 40% in general. According to the author's investigation [9,10], the morbidity of cysticercosis pisiformis is as high as 25% for 5,000 rabbits slaughtered in a small abattoir. The undernourishment, emaciation, lower resistance, inferior quality of meat and low quality of skin and hairs are showed in the rabbits infected with cysticercus pisiformis. If seriously infected, the rabbits, especially in the young ones, are able to death in large quantities, which inflict significant economic loss.

The scolex is a main organ of taenia pisiformis to attack the host. It is very important to understand the ultra-structure changes of scolex for prophylaxis and treatment of cysticercosis pisiformis. Similar to cysticercus cellulosae [11-13], the scolex of cysticercus pisiformis also has the rostellum and the sucker. But now, there is little research on the morphologic ultrastructure of the scolex. Many ultrastructure changes of the scolex are not completely clear. The author used scanning electron microscopy (SEM) to observe detailedly ultrastructue changes of the scolex that located in the cyst or evaginated from the cyst after the cultivation. The different ultrastructure changes of scolex in the relative rest and motion states were found out in this research. The results are to be shown as follows.

Material and methods

Collection of cysticercus pisiformis

The cysticercus pisiformis were gathered mainly from rabbit-kill abattoir. When the rabbit with cysticercus pisiformis was found in the inspection of paunch, the well-developed cyst located on the greater omentum was clipped immediately by sterilized surgical scissor, then put them in sterilized Petri dish, and took them to laboratory for standby.

Treatment of cysticercus pisiformis

In order to observe the ultrastructure of the csolex of cysticercus pisiformis in the relative rest and motion states, the several different methods were used to process the cysticercus pisiformis. For example, when the relative rest states of the scolex was observed the cyst was cut by

ophthalmic scissors. Cystic liquid were flowed from the cyst. The scolex, neck and strobila were collected from the cystic wall carefully, put them into physiologic saline solution and slightly rinsed to remove the cystic liquid that was adherent on the surface of scolex. Finally, the scolex, neck and strobila after rinsing were put into 2.5% glutaraldehyde for the fixation.

Cultivation of cysticercus pisiformis

In order to observe the relative-motion states of the scolex of cyticercus pisiformis, according to previous methods [14 – 16] and improving, it was that the well-developed cysts were put into physiological saline solution that contained 20% rabbit bile and preheated in 37℃ and cultured in 37℃ incubator. After culturing for 12 h, when the scolex, neck and strobila were evaginated from the cysts they were put carefully into physiological saline solution by ophthalmic tweezers to fully rinse, and then fixed with 2.5% glutaraldehyde. For a few of cysticercus pisiformis that could not evaginated from the cyst autonomously, their cysts were cut by ophthalmic scissors to help the scolex, neck and strobila to evagination by artificial method.

Preparation of the scanning samples

The scolices fixed well with 2.5% glutaraldehyde were rinsed fully with PBS (pH 7.2) in twice for 5 min respectively, then, sucked the PBS liquid dry on the surface of scolex with absorbent paper. According to what to be observed, that part of the scolex would be fixed on sample stage with conductive adhesive under anatomic microscope. The samples were observed and recorded with Quanta type 200 scanning electron microscopy (manufactured by FEI Company of American).

Results

Ultrastructure of the scolex located in the cyst

Most of the scolice in cyst were in the relative rest states, or had a very little the movement. Viewed from the top of the scolex, the tegument muscular column and tooth-hook of the rostellum looked like an umbrella that covered the top of the scolex (Fig. 1A). The branch of tooth-hook was in the upright and formed the false permutation as two rows of tooth-hook. There was a carinal nodule-like structure where the tooth-hook and tegument muscular column were connected. Viewed from the side of the scolex, the rostellum located in the front end of the scolex. The front end of rostellum was dull and joined by the chicken-claw-like tooth-hook through the tegument muscular column. The sucker located behind the rostelleum and presented around the scolex by the equidistance. The tooth-hook of the rostellum lined up only in one row. Each tooth-hook had an antler-like branch (Fig. 1B), and formed a pair of longer and shorter hooks. The front of longer hook was sharp, and looked like chicken claw. The shorter hook was dull, and looked like human beings' thumb。The surface of tooth-hook was smooth, and full of sheen with the hard appearance. The sucker looked like a round cave. The edge surrounding the round cave was thicker, rougher and more protuberant from the surface of the scolex. The tegument surface of the cave was coarser, but the cave wall of the sucker was smoother. The depth of the cave was dependent on its states. It was deeper when the cave was contracting, but was shallower when relaxing. The rostellum would appear contractive-like reaction when it was stimulated by certain factors. At this time the front of rostellum became the flat states. The front end of rostellum formed seven orange-petal-like structures that contained three pairs of tooth-hook in each. The apophysis of tegument muscular column was obvious. The tooth-hook of the rostellum began to abduction. The ring-like contraction of sukers emerged in the cave periphery. The suckers became smaller and extruded from the surface of the scolex.

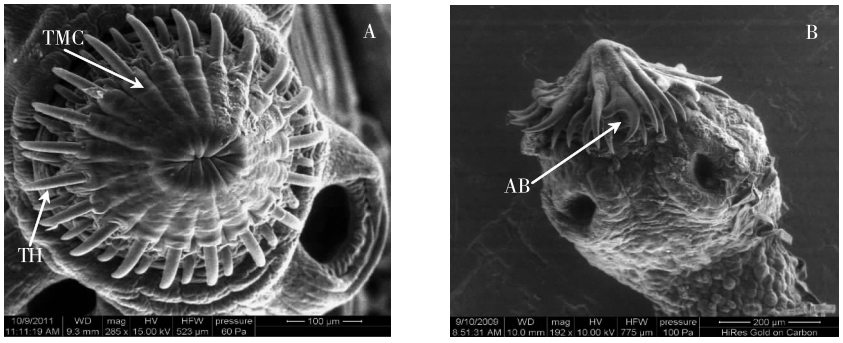

Fig. 1 The scolex is in the relative rest states

A. The tegument muscular column (TMC) and tooth-hook (TH) of the rostellum looks like an umbrella (×285).

B. The rostellum with antler-like branch (AB) is located in the front end of the scolex and the sucker is located behind the rostelleum (×192).

Ultrastructure of the scolex evaginated from the cyst

After being cultured with 20% bile medium the scolex evaginated from cyst was in the different motion states. When the scolex evaginated from cyst, viewed from the side of the scolex, the proximal rostellum moved forward, the stem base of rostellum widened broaden and the tooth-hook on the rostellum spread out or apart. The sucker appeared ring-like contraction and protruded distinctly from the surface of the scolex. Viewed from the top of the scolex, the tooth-hook on rostellum began to stretch and gradually extended out from the states of attaching to the rostellum. At this time it was easily observed that the tooth-hook shaped like antler branch (Fig. 2A). Following the contracting of rostellum there was a revolving change in the front end of the rostellum. With the strong contraction of tegument muscular column the tooth-hoods almost completely stretched and the antler-like branch structure was more visible. At the same time, the suckers also contracted and protruded from the surface of the scolex. The contraction of sucker had two kinds of pattern. One was ring-like contraction. There were two or three larger and lots of smaller ring-like creases around the sucker. When the ring-like contraction of sucker was strong the sucker looked like a wheel-like structure (Fig. 2B). The other was a longitudinal-like contraction that made the portal of sucker smaller and smaller, even to close.

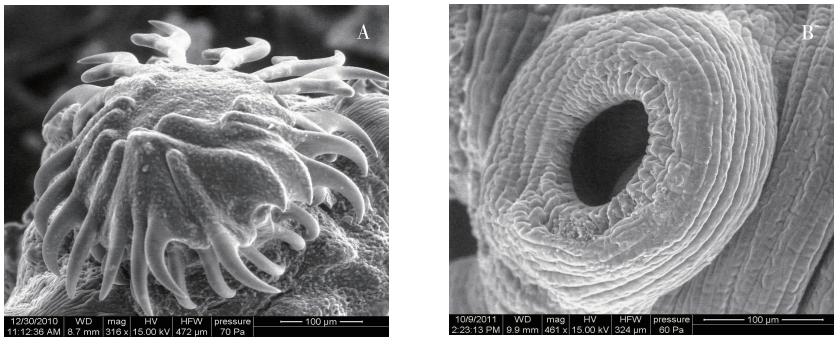

Fig. 2 The scolex is in the relative motion states
A. With the strong contraction of tegument muscular column the tooth-hook almost completely stretches and the antler-like branch structure is more visible(×316).
B. When the ring-like contraction of sucker is strong the sucker looks like a wheel-like structure(×461).

Discussion

Ultrastructure and function of tooth-hook

So far, the rostellum of cysticercus pisiformis as same as cysticercus cellulosae were reported to have two rows of small hooks in some textbooks [17, 18] and research papers [19, 20]. That is only morphologic structure of the rostellum of cysticercus pisiformis by squash slide under light microscope. By SEM it was found that the tooth-hook on the rostellum was not two rows, but only one row. The tooth-hook looked like antler and had a branch connecting two hooks with different length, in which one was longer and other was shorter. For this reason, the two row hooks observed under light microscope were only a kind of pseudo-stratified arrangement, but actually a result that antler-like hooks were broken by squash slide. The surface of hooks was smooth and rich in burnish feeling under SEM. This result was the same as one reported in squash slid stained with hematoxilin and eosin (HE). When squash slides were stained with HE a lot of calcium salt was detected on hooks [9]. Because the hooks were full of calcium salt ingredients, they become harder and turned into a main attack organ of cysticercus pisiformis. After being cultured, the scolex evaginated from the cyst was observed by SEM and its hooks of the rostellum were discovered to motion in different degree. When the tegument muscular columns at the front of the rostellum contracted a pair of anlters-like tooth-hook were sticking out, which greatly improved the invasiveness of rostellum to the host tissues.

Ultrastructure and function of sucker

The sucker is also an invasive organ of the scolex of cysticercus pisiformis and has the function of helping the scolex to fix. The movement of sucker also had some characteristic. In the relative rest states the sucker looked like round cavernous structure. Its ambitus was thicker and slightly protruded from the surface of the scolex. When the scolex was in the motion states, the tooth-hooks of the rostellum were extending and the suckers contracting. The characteristics of the sucker motion was that it was not only to do ring-like movement that made the cave of sucker to present wheel stratiform and gradually got smaller and smaller until completive

imperforation, but also to make longitudinal contraction that make sucker to have more adsorptive power. The sucker obviously protruded from the surface of the scolex while it was in the contraction. The four suckers of the scolex became the four major tubercles, and was in the states of invasion. This movement was very helpful for the suckers to adsorb the host tissues strongly. According to the reports [17, 19, 21, 22], the muscular fibers of the sucker are extremely developed. It has ring-like muscular fibers and also had radial longitudinal muscular fibers, so the sucker could make both ring-like and longitudinal-like contraction. The morphologic changes of sucker were the results of the contraction of its muscular fiber. From the analysis of morphology and structure the sucker might have the absorptive function except to the function of adsorption and fixation. This function could help the material interchange between polypide and host. Liking newborn animal suck the breast, cysticercus pisiformis could draw nutrient substances from host through sucker that was fixed to host tissues.

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The Effect of Rubber Flooring on Gilt Skin Bacterial Count

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Summary: The aim of the study was to investigate the effect of rubber flooring on the gilt skin microbiological cleanness. The study was carried out at a commercial pig breeding farm service unit during autumn and winter production cycles, including two groups of 14 gilts each, kept in individual stalls with slatted concrete floor for 28 days. Group 1 gilts served as controls, while in group 2 the stall floor was covered with adjusted textured rubber mat. On cycle days 1, 8, 15 and 28, swabs were obtained from the gilt skin surface and total aerobic mesophilic bacteria (AMB) were enumerated. Study results revealed the mean AMB count on all days observed and AMB count on day 28 to be significantly higher ($P < 0.05$) in the experimental *versus* control group in both cycles. Also, a significantly higher ($P < 0.05$) AMB count was determined in the experimental group on days 8 and 15 during winter. In conclusion, the use of rubber flooring for gilts may result in increased values of total AMB on their skin.

Introduction

Animal welfare and productivity depend on the flooring type used in their housing [1, 2]. The type of flooring also influences animal house hygiene and initial cost of house construction. Breeding females in intensive pig production are mostly kept on fully or partially slatted concrete floors, which are in many ways detrimental for their welfare, wherefore alternative housing systems, such as rubber flooring, are gaining interest [3].

Rubber flooring may provide welfare benefits to breeding females in terms of lesion reduction and improved lying comfort, at lower temperatures in particular [3, 4]. To our knowledge, however, studies investigating visual or microbiological cleanness of the skin of gilts housed on rubber flooring have not yet been reported. Therefore, the aim of the present study was to assess the effect of rubber flooring on the gilt skin microbiological count.

Material and methods

The study was carried out at a commercial pig breeding farm service unit in cooler seasons, during autumn and winter production cycles. Each cycle included 28 gilts kept in individual stalls (size 1.80 m × 0.60 m) with slatted concrete floor (slat width 8 cm, slot width 2 cm) for 28 days, a period that breeding females are kept in individual stalls post-mating. The gilts were divided into two equal groups. Group 1 gilts served as controls. In group 2, the stall floor was covered with 2-cm textured rubber mat (GUMIIMPEX Inc., Varaždin, Croatia), adjusted to the stall size and floor slots. Rubber mats were interconnected using the puzzle pattern and attached to the concrete floor. Upon completion of a study cycle, the mats were disconnected, cleaned and disinfected, and then placed again before the next cycle. Gilt cleanness was assessed by taking and analyzing swabs from their belly skin. On cycle days 1, 8, 15 and 28, 5 cm × 5 cm swabs were obtained from the skin surface of the ventral part of the gilt mesogastric region and total aerobic mesophilic bacteria (AMB) were enumerated after 37℃/24 h incubation. Results were expressed as $\log_{10}$ CFU/mL of rinse fluid. Statistical data processing was performed by the STATISTICA v. 10 (Statsoft Inc., 2011) software using descriptive statistics methods, Kolmogorov-Smirnov test, Student's t-test and ANOVA Repeated Measures.

Results and discussion

Floor cleanness of animal housing and thus of animals is influenced by numerous factors such as the size, construction and type of flooring material, cleaning methods and frequency, specific climatic conditions, type of feed and feeding methods, animal behavior and manure texture, etc. [5 – 7]. Considering floor cleaning conditions, slatted floor is certainly preferred over solid floor, but controversy arises related to animal welfare [8].

In the present study, the floor in the experimental group housing was covered with rubber mats. Study

results showed the AMB count determined on day 28 *versus* day 1 of both cycles to be significantly lower on the skin of control gilts ($P < 0.05$), whereas in experimental group it was higher, yet not reaching statistical significance ($P > 0.05$) (Fig. 1 and 2).

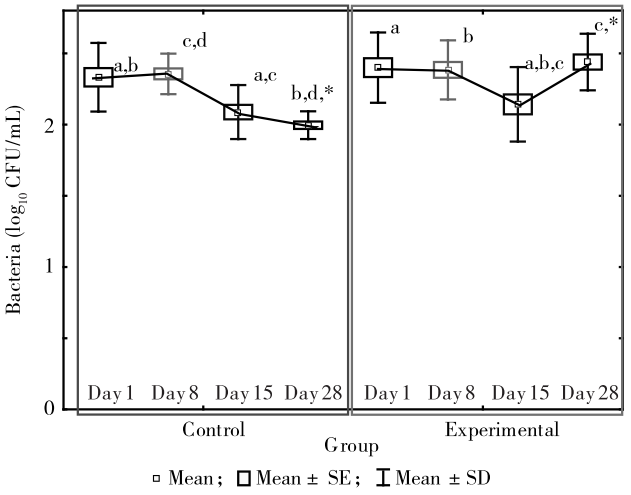

Fig. 1 Aerobic mesophilic bacteria count on the gilt skin in autumn cycle

[a,b,c,d] Values between days within a group marked with the same letter differed statistically significantly at the level of $P < 0.05$.

[*] Values between the groups differed statistically significantly at the level of $P < 0.05$.

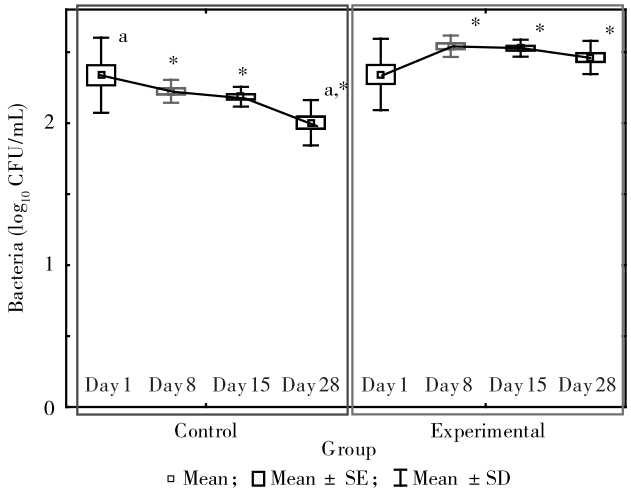

Fig. 2 Aerobic mesophilic bacteria count on the gilt skin in winter cycle

[a] Values between days within a group marked with the same letter differed statistically significantly at the level of $P < 0.05$.

[*] Values recorded on the same days in different groups differed statistically significantly at the level of $P < 0.05$.

Comparison of AMB count between the control and experimental groups in both production cycles revealed

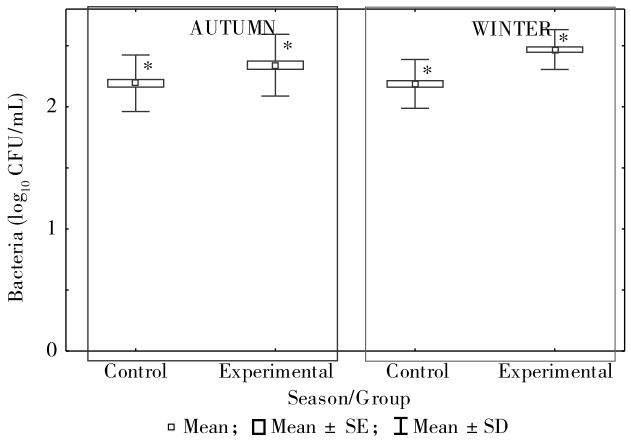

Fig. 3 Mean aerobic mesophilic bacteria count on the gilt skin of control and experimental groups

[*] Values recorded in the same season differed statistically significantly at the level of $P < 0.05$.

the mean AMB count on all days observed and the AMB count on day 28 to be significantly higher ($P < 0.05$) in the experimental group (Fig. 1 – 3). Moreover, a significantly higher ($P < 0.05$) AMB count in the experimental group was determined on days 8 and 15 during winter (Fig. 2). The higher AMB count on the skin of experimental group gilts could be ascribed to the plug-like pattern of the floor rubber mats, which results in feces residues and/or improved lying comfort and longer gilt lying position [3, 4] and thus their prolonged contact with feces.

Conclusions

The present study demonstrated the use of rubber flooring to increase total AMB count on individually housed gilt skin during autumn and winter production cycles in service unit.

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Postparturent Haemoglobinurea in Cattle, Buffaloes and Its Relation with Some Serum Elements

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Summary: Post parturient hemoglobin urea, It is a one of the most important metabolic disease in veterinary medicine, this problem is common in Egypt where small farmers cattle feed on unbalanced ration and by the end of winter were they fed on Egyptian clover, *Alfa Alfa*, it is rich in calcium, this resulted in deficiency of phosphorus.

Serum mineral Concentrations are highly dynamic around the time of calving as haemostatic mechanism.

The present study was conducted on thirty Egyptian native breeds cows and buffaloes, they exhibiting signs of red urine, loss and depraved appetite, pica as licking of hair coat of other animals and chewing of inanimate objects especially bones, rough hair, decrease in milk production, and in some cases sternal recumbence, and having inorganic phosphorus of plasma below critical level of normal serum inorganic phosphorus 4. 5 mg/dl.

The objective of this study was to explore the relation between post parturient hemoglobin urea, serum level of inorganic phosphorus, and some elements has relations with phosphorus metabolism.

Key words: post parturient hemoglobin urea, hypophosphatemia, phosphorus deficiency, metabolic diseases, Egypt

Introduction

Parturient haemoglobinuria is a disease of economic importance in buffalo rearing countries in general and in India, Pakistan and Egypt in particular (Pirzada and Hussan, 1998; Radostits, et al, 2006), Reported that, the clinical findings of Hemoglobinuria in cattle are inappetence, and weakness develop suddenly and there is a severe depression of the milk yield, although in some less acute cases, the mucous membranes are pallid, and the cardiac impulse and jugular pulse are much augmented. A moderate temperature rise (40℃; 103.5°F) often occurs. The feces are usually dry and firm. Dyspnea may be obvious and tachycardia is common.

Durani, et al (2010) reported that the highest prevalence was seen in buffaloes suffered from post parturient haemoglobinuria at the 5th lactation. The disease was usually associated with feeding of Egyptian Clover(Berseem) from November to May and of (Awad and Abdel-Latif, 1963; Abdel-Latif and Awad, 1964). Haemoglobin urea occurred in 12 river buffaloes in West Azerbaijan, Iran. The affected buffaloes were aged 5 to 10 years(Dalir-Naghadeh, et al,2006).

Decreased concentration of inorganic phosphorus before calving causes increase risk of deficiency after calving with symptoms of post parturient Hemoglobinuria (Mordak and Nicpon, 2006). The dietary-P deficiency along with very wide Ca: P ratio may result in decreased phosphorus absorption from the intestinal tract leading to Hypophosphatemia and which may cause Hemoglobinuria (Jain, et al, 2012). The ingestion of cold water or exposure to extremely cold weather may precipitate an episode of Hemoglobinuria. In an affected herd may have serum inorganic phosphorus levels within the normal range (Radostits, et al, 2006)

Durani, et al (2010) = (21) reported very low levels of serum phosphorus (0.4 – 1.5 mg/dL) during the hemolytic crisis. During post parturient Hemoglobinuria, the serum inorganic phosphorus significantly, 3.68 ± 0.95 mg/dl. The serum phosphorus level in early stage of lactation (4.64 ± 0.53 mg/dl) of buffaloes. And there was drop in calcium level during early stage of lactation (8.19 ± 0.83 mg/dl) than the normal healthy buffaloes (11.21 ± 0.19 mg/dl) (Hagawane, et al,2009).

Were significantly lower in PHU-affected buffaloes in serum, calcium (mg/dl) was 9.8-t 1.0, 9.9-t 1.3, phosphorus 5.41-t 0.6, 1.9-t 0.6, copper (mg/dl) 118.4-t 5.2, 65.4-t 6.0 in healthy and diseased buffaloes respectively. (Akhtar et al., 2007a). Hagawane, (2009), Calcium (mg/dl), Early lactation 8.1 ± 0.83a, Mid lactation 9.65 ± 0.78a, Dry (pregnant) 7.81 ± 1.02a, Healthy control 11.21 ± 0.19b, f-value 3.44s, phosphorus (mg/dl) early lactation 4.64 ± 0.53a, mid lactation 5.18 ± 0.44a, dry (pregnant) 4.75 ± 0.51a, healthy control 6.55.

Akhtar, et al, (2007b) Serum phosphorus, copper and selenium were significantly ($P < 0.001$) lower, whereas potassium, iron and molybdenum ($P < 0.001$) were higher in buffaloes suffering from PHU than healthy buffaloes. Calcium concentration did not vary between

two groups.

Material and methods

The present study included 40 animals, 10 Egyptian buffaloes, 10 mixed breeds cows, there ages round 5 to 10 years, no of previous lactation was 3rd to 4th stage of lactation, after parturition and some of them pregnant in 3rd to 4th month, and 5 bulls, were appeared red urine, drop in milk production, were selected randomly from fifteen villages in Qena governorate.

Results

Clinical examination, The temperature was normal and may be slightly increase to 38.2℃, Pale mm, anemia, tachycardia, rapid and shallow breathing, characteristic stretching in feces.

The disease appears suddenly and all of them appeared red urine, the appetite differs from normal, moderate and off food in advanced stage. Weakness, depression, and drop in a milk production. The most cases, diseased in winter season, especially in January, February, after that, and this related to feeding on Egyptian clover. (Berseem), sporadic cases all year, return to other type of Berseem grow all over the year in Egypt it is Hejaz Berseem in reclaimed land in desert, but Egyptian clover (Berseem) only in winter.

Most of them response to treatment of HU as phosphorus compound, but some died. Little number recovers spontaneously without medical interference. The objective of this study was to assess the of some serum biochemical parameters as cause of haemoglobin urea in cattle and buffaloes. Biochemical results as in Table 1.

Table 1 Values of concentration of , Phosphorus (P), Calcium(Ca) Copper (Cu) and Iron (Fe) in blood serum of animals suffered from haemoglobin urea

Animal	No	P	Ca	Cu	Fe
Pregnant Cows	5	1.37 mg/dL 0.443 mmol/L	7.54 mg/dL 1.885 mmol/L	105.14 μg/dL 16.51 μmol/L	98.17 μg/dL 17.57 μmol/L
Cows after Parturition	5	4.72 mg/dL 1.525 mmol/L	9.5 mg/dL 2.375 mmol/L	120.28 μg/dL 18.88 μmol/L	106.62 μg/dL 19.08 μmol/L
Pregnant Buffaloes	5	0.66 mg/dL 0.213 mmol/L	7.27 mg/dL 1.817 mmol/L	100.67 μg/dL 15.81 μmol/L	92.44 μg/dL 16.55 μmol/L
Buffaloes after Parturition	5	3.27 mg/dL 1.056 mmol/L	10.0 mg/dL 2.5 mmol/L	78.11 μg/dL 12.26 μmol/L	117.71 μg/dL 21.07 μmol/L

Discussion

High occurrence of Hemoglobinuria in winter especially in 4th and 3rd lactation, ages and role of Berseem as main cause of disease and Clinical signs agree with (Akhtar et al, 2006; Abdel-Latif and Awad, 1964) decrease of phosphorus, normal concentration of calcium, and Iron may be normal or increase in blood serum of bovine affected with Hemoglobinuria agree with Akhtar et al (2007), Durrani et al (2010).

Conclusion

Bovine suffer from haemoglobin urea especially in winter when feed on Berseem where it is high in calcium, low phosphorus and consider as a cruciferous plant. Blood serum of diseased animals, decrease in concentration of phosphorus, and highly decrease in pregnant cows and buffaloes than those after parturition. Calcium was in normal values. Copper was decrease in some and normal in others. But Iron was normal or increase.

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Acquisition of Drug Consumption in Pig Production in Austria

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Summary: In recent years, antibiotic usage in veterinary medicine has been under discussion because of the development of antimicrobial resistances, which could limit human treatment possibilities. Quantification of drugs used in veterinary medicine is therefore of great public concern and importance. Exact measuring of veterinary antimicrobial consumption is challenging however. In this paper a bottom-up study is contrasted to the European Surveillance of Veterinary Antimicrobial Consumption Project ESVAC [1,2], which follows a top-down approach to measure antibiotic usage. ESVAC registers the quantities of antimicrobial products sold for treating food-producing animals in the European member states. Unfortunately, it doesn't give any information of antimicrobial consumption on animal species level. Also, results are expressed in total weight of antibiotic substances consumed per Population Correction Unit (PCU), which may lead to false interpretation, as therapeutic potency differs from one antimicrobial substance to another one. In this study, drug application data were collected in the context of a quality-securing system in an Austrian meat production company. The ambition was to develop a pilot bottom-up system for veterinary drug application monitoring.

Introduction

Antibiotic consumption and resistances

Since the discovery of Penicillin in 1928 the treatment possibilities in veterinary as well as in human medicine have been drastically extended by the usage of antimicrobial substances. However, the use of antibiotics causes the occurrence of bacteria insensitive against some antimicrobial substances. This antimicrobial resistance entails a limitation of the availability of effective antibiotics, which often has prolonged disease duration, high costs implications and in the last resort death as consequences. An association between the quantity of antimicrobial substances used in medicine and the occurrence of resistances has been established [3 – 5]. As most of the antibiotics used in veterinary medicine are structurally analogous or even identical to those used in human medicine, the copious application of antibiotics in livestock husbandry is strongly criticised. The European Commission, the Food and Agriculture Organization (FAO), the Office Internationale des Epizooties (OIE) and the World Health Organization (WHO) recommend monitoring programs for the surveillance of antimicrobial consumption in veterinary medicine.

European surveillance of veterinary antimicrobial consumption (ESVAC)

ESVAC was initiated in 2009 by the European Commission, who asked the European Medicines Agency to collect and to interpret antibiotic sales data from European Countries [1]. The aim of the project was to develop a standardized method of data collection in order to evaluate antibiotic sale trends over time. Since the launch, two ESVAC reports have been published, the recent one illustrating the antimicrobial consumption in veterinary medicine in 19 European countries in 2010 [2].

Situation in Austria

In Austria, article 6(3) of the national Zoonosis Act [6] requires the implementation of a surveillance system for the usage of antibiotics. Feasibility studies on estimating antimicrobial consumption in cattle, pig and poultry production have been successfully conducted and the necessary methodology has been published [7, 8]. The dispensary of veterinary medical products is statutory regulated by the Animal Health Service Regulation 2009 [9]. Each drug dispensing, drug return and drug application has to be officially documented. In special cases, when disease treatment requires a repeated or long-term drug application, the dispensary of drugs from the veterinarian to the farmer is allowed. The aim of this study was to check the plausibility of bottom-up drug application data originating from a quality-securing system of an Austrian meat production company in order to potentially complement top-down data and to provide a plausibility estimate for such data.

Material and methods

Since many years, the company providing the data has implemented a strict quality-securing-system along the food chain from its contract farms to slaughterhouse, with

computer-aided supervision from the animal up to the food product. At farm level, farmers record each drug application via an online platform. During slaughter meat inspection, data about the health status of pigs is collected and reported back to the originating farm and its veterinarian in charge in order to facilitate improvement measures. For this study, drug application data from 76contractfarms was evaluated in a time period from January 2008 to December 2011. Data included information of i) farm registration number, ii) treatment date, iii) drug name, iv) drug authorisation number, v) treatment duration, vi) treatment frequency per day, vii) therapy indication, viii) swine age category (suckling pigs, feeder pigs, fattened pigs, sows, and boars), ix) number of animals treated, x) quantity of applied drug per animal and xi) quantity of total applied drug. To each drug application entry, the active ingredient(s) and the corresponding ATCvet Code (Anatomical Therapeutic Chemical classification system) [10] were allocated. Further analysis was focussed on antimicrobial products. Finally, the total quantity of antimicrobial substance was calculated and expressed in number of Animal Daily Doses (nADD) and in number of Prescribed Daily Doses (nPDD) [7, 12]. In order to permit the comparison between animal species, results were referred to the animal bodyweight, expressed in livestock unit (LU) [7].

Subsequently, data and results were checked for plausibility. For this purpose, a comparison between antibiotic application quantities and dispensary quantities is in progress.

Results and discussion

The 2nd ESVAC report [2] compiles the quantities of antimicrobial products sold for treating food-producing animals in European member states in the year 2010. Data of antibiotic sales were obtained from wholesalers, marketing-authorisation holders, pharmacies and feed mills. For evaluation, veterinary antimicrobial products were allocated to their ATCvet-Code [10] and categorized according to their pharmaceutical form ("intrauterine preparations", "intramammary preparations", "bolus", "oral paste", "injection", "oral solution" "oral powder" and "premix"). The population correction unit(PCU) was introduced in order to refer the total amounts sold to the size of the animal population at risk and to permit comparisons between countries. Results are presented in mg/PCU per country, antimicrobial class, pharmaceutical form and number of active ingredients.

ESVAC is based on a harmonized data collection form and permits a comparison of sale patterns between the 19 different countries. Unfortunately, the project does not give any information on antimicrobial consumption per animal species or age class. Also, the treatment indication and the diagnosis made by the veterinarian are not included. Furthermore, results of ESVAC are expressed in total weight of antibiotic substances consumed per PCU. However, the therapeutic potency differs between the antimicrobial agents. Comparing the total of antibiotic weight consumed between countries may lead to a false interpretation, as some antimicrobial substances require very high doses to have the same effectiveness as other substances. For example, a country mainly using tetracycline for animal treatment may show a very high antimicrobial consumption when represented in weight unit.

The study at hand aims to quantify antimicrobial consumption in Austrian pig production using the international measure unit nADD and nPDD [12]. As data are collected directly by the farmer, they contain detailed information, in particular regarding quantities of drug administered, animal species, animal age class and therapy indication. This allows a very specific, deep going analysis of drug consumption. However, preliminary results show incomplete and inconsistent records in dispensary and application documents, in particular concerning drug authorisation number, drug quantity, treatment indication and animal category. In consistencies between the quantities of antibiotic products dispensed from veterinary to the farmer and the quantities of applied antibiotics recorded by the farmer were observed.

Table 1 summarizes the differences between the ESVAC and the study at hand, illustrating the respective advantages and limitations.

ESVAC well illustrates the quantities of antimicrobial products generally sold in 19 countries of the European Union. Unfortunately, as most of the antimicrobial products are accredited for more than one animal species, drug consumption per animal species cannot be determined in such a broad top-down analysis. For a more detailed antimicrobial consumption analysis, a specific data collection of drug applications including the treated animal species, age category and the therapy indication, as it was achieved in this study, needs to be realized. Furthermore, expressing the quantities of antimicrobial agents consumed in weight of active ingredient is not an optimal solution. Instead of that, drug use should better be expressed in treatment incidence [11] or in nADD and nPDD [7, 12]. The detailed drug application collection method of this study permits a quantification of the antimicrobial consumption expressed in nADD or nPDD per animal species, animal age class and including administration indications. Though, the inconsistences in data show that regulations alone are not a guaranty for the correct implementation of

Table 1 "ESVAC Project" (European Surveillance of Veterinary Antimicrobial Consumption) and the study at hand in contrast (nADD: number of Animal Daily Doses; nPDD: number of Prescribed Daily Doses)

	ESVAC Project	The study at hand
Analysing system	Top-down	Bottom-up
Data collection	Overall national sales data	On-farm records
Quantification per animal species or age class	No	Yes
Information about therapy indication/diagnosis	No	Yes
Expression in nADD, nPDD	No	Yes
Comparison between countries	Yes, but limited because consumption is expressed in weight unit	No, since no harmonized data collection form is established yet
Advantages	Harmonized data collection, comparison between countries	Consumption at animal species level, information about therapy indication, expression in nADD, nPDD
Limitations	No Information on animal species and therapy indication	No harmonized data collection, inaccuracy in data entries

documentation rules.

Conclusions

Collecting drug application data recorded by the farmer in the course of each single treatment (bottom-up approach) is the method of choice to analyse the antimicrobial consumption in veterinary medicine. However, further analyses are necessary in order to establish a secure and harmonized data collection form.

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Livestock Biosecurity—Past, Present and Future

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Summary: As the world travel increases and global agriculture evolves, the potential for spreading infectious diseases amplifies, ratcheting up the importance of biological security to new levels. Every farmer has the ultimate responsibility to protect the health of animals under their care and should seriously consider the development of a biosecurity program for their herds. This can be accomplished by working in close cooperation with vets, extension specialists, as well as state officers.

The aim of the project is to define the main areas of biosecurity as an important part of preventive measures in farm animals, develop a basic livestock management strategy aimed at preventing the introduction of pathogens into herds or the spread of pathogens within a herd.

The goal of a biosecurity plan is to reduce the risk of disease exposure. A disease outbreak occurs when animals are exposed to an infection and the environmental conditions and animal's resistance are at a certain level. We can influence the occurrence of disease by identifying critical control points or changing risk factors between these points. Thorough cost analyses enable to find an optimum point at which the treatment or in some cases prevention of disease brings returns higher than the costs incurred by the disease.

An appropriate hygiene standard is a prerequisite for maintaining good health and a high level of productive and reproductive performance of farm animals. Compliance with the general principles of biosecurity is a prerequisite for production of healthy and biologically wholesome raw materials and foodstuffs of animal origin as one of the important indicators improved competitiveness and economic viability of livestock.

Introduction

As the world travel increases and global agriculture evolves, the potential for spreading infectious diseases amplifies, ratcheting up the importance of biological security to a new level. Every farmer has the ultimate responsibility to protect the health of animals under their care and should seriously consider the development of a biosecurity program for their herds. One of the most important critical points for maintaining the good health of livestock is a hygiene program. The prevention of disease introduction and the reduction in the huge amount of microorganisms in animal houses to an acceptable level have to be the objectives of a biological security program. A suitable biosecurity program helps to lower the risk of pathogens being transferred from farm to farm. The assessment of health status of livestock and implementation of biosecurity measures will protect the farm from diseases. The biosecurity plan should be reviewed regularly and amended as the situation on the farm changes or new knowledge is acquired. This can be accomplished by working in close cooperation with vets, extension specialists, as well as state officers.

Material and methods

The aim of the project is to define the main areas of biosecurity as an important part of preventive measures in farm animals, develop a basic management strategy for livestock aimed at preventing the introduction of pathogens into herds or the spread of pathogens within a herd. A complex of preventive measures, designed to prevent the penetration of infectious agents into the farm, was put together and analysed.

Results and discussion

Good management of livestock should be based on two fundamental principles-the fulfilment of fundamental animal needs and biosecurity practices. Already in the second half of the 20th century the introduction of measures to prevent importing infection in the farm was an integral part of responsibility of the State Veterinary Administration's local authorities, which proceeded in accordance with applicable regulations (standards) and expert opinions while performing an assessment of project documentation on farm livestock constructions, and assessed constructions from animal hygiene and epidemiological viewpoints with regard to various factors, including the natural foci of diseases.

Veterinary and sanitary requirements applied to the choice of construction site, availability of adequate sources of feed and water, and a distance from residential areas. The following parameters were determined:

– sanitary protection zones of farm, taking into account the number and species of animals kept on the

farm, including the assessment of farm impact on the surrounding environment;

– farm protective zones from common public facilities (roads, railways, etc.);

– recommended distances between residential buildings and stables for livestock.

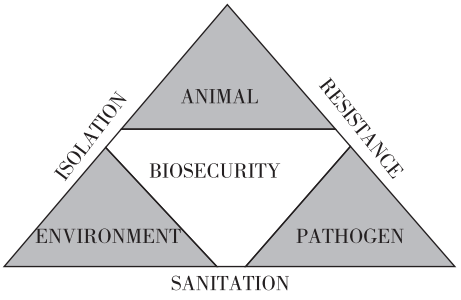
It is important to employ measures such as entrance disinfection and quarantine to prevent an introduction of disease into the farm via animals, people, vehicles, feed, water and waste products, to be able to run a closed herd system. An integral part of veterinary and sanitary protection is a division of a farm into the white zone (buildings for animal housing) and black zone (auxiliary operations). In order to protect the animals against the spread of infection within the farm, all-in all-out principles of operation, regular sanitation of stables and adjacent areas, isolation of sick animals or animals suspected of being infected, principles of dead animal disposal including hygiene handling of waste should be introduced.

At present, from a practical viewpoint biosecurity can be divided into an external and internal part.

External biosecurity is a management strategy aimed at minimizing the penetration of micro-and macro-organisms that can cause disease in animals on a farm or in stables. External biosecurity measures include prevention of infectious agent penetration in the farm, limited access of visitors and setting up barriers, development and tightening up of rules for transporting, protection from other animals species (wild animals, birds, insects, rodents), animal protection zones. From an economic point of view, the principles of external biosecurity are a prerequisite for achieving farm profitability and high quality of final products.

Internal biosecurity can be understood as a set of preventive measures targeted to reduce the microflora within an operation (i.e. prevention of stable fatigue, or else stable microbism). These include optimizing production technology systems, with an emphasis on closed herd turnover, creating barriers – black and white zoning of the farm, sanitation measures – disinfection, insect and rodent control, vaccination programs appropriate for a given species and production stage of animals, regular monitoring of health status of animals aimed at detecting endo- and ecto-parasites with a view to intermediate hosts, control of raw materials and products including the inspection of slaughtered animals to get objective information about the health of food-producing animals and epidemiology in their place of origin, monitoring of herd with emphasis on a strict observance of herd health program (including medical tests and examinations), sanitation routines, establishment of the HACCP system. This is important especially in livestock units with high concentrations of animals, with fast turnover of animal population or repopulation of parent herd, where there is a high pressure of stable space and its surrounding area, which has a negative impact on production and reproductive breed performance, growth and health of farm animals.

Disease occurs when animals are exposed to an infection and the environmental conditions and animal's resistance are at certain level. We can influence the occurrence of diseases by identifying critical control points or changing risk factors between these points. Thorough cost analyses enable to find an optimum point at which the treatment or in some cases prevention of disease brings returns higher than the costs incurred by the disease.



As herd size increases and animals are placed in more intensive housing management systems, it is easier for infectious diseases to enter and spread throughout the herd. In agreement with Buhman et al. (2000) biosecurity management practices are designed to prevent the spread of disease by minimizing the movement of biologic organisms and their vectors (viruses, bacteria, rodents, flies, etc.) into and within the farm. Biosecurity consists of three major components: isolation (a strategy to keep infectious agents and discharges away from susceptible animals), resistance (nutritional, environmental, pharmacological and immunological practices that improve the animal's ability to resist disease) and sanitation (key factor in minimizing spread and limiting the course of infectious diseases-disinfection of any potentially contaminated equipment or facilities) focused on the complex influence of pathogen – animal – environment interaction.

Conclusions

Appropriate hygiene standards on a farm are a prerequisite for maintaining good health and a high level of productive and reproductive performance of farm animals. Compliance with the general principles of biosecurity is a prerequisite for production of healthy and biologically wholesome raw materials and foodstuffs of animal origin as one of important indicators of improved competitiveness and economic viability of a livestock

operation.

An effective and well-planned biosecurity plan on a farm is an important part of the herd (flock) health program to secure sustainable production. The stock manager, along with the veterinarian should strive to develop and implement a biological safety plan tailored to the farm as part of the overall management strategy of health, production and reproduction.

From the hygiene point of view the complex of farm animal biosecurity can be summarized as the following ten golden rules:

1. Suitable farm location
2. Closed herd (flock) system
3. Control of entry and movement of persons in the farm premises
4. Control of vehicle movement in the farm
5. Black and white zones
6. Optimization of technological systems
7. Feed and drinking water hygiene
8. Disinfection, insect and rodent control and deodorization
9. Targeted prophylaxis, diagnosis and therapy
10. Herd health management

Acknowledgement

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Apoptosis of Lymphocytes and Brain Tissue Cells Caused by Canine Distemper Virus

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Summary: To research apoptosis of lymphocytes and brain tissue cells, this investigation was performed on 16 dogs infected by canine distemper virus and 6 health dogs as control. The lymphoid and brain samples were collected by the necoropsy. Then, the paraffin sections were made and stained by HE, and immunohistochemistry, that was anti-CDV sABC assay, and TUNEL assay. The results demonstrated that CDV had varying degree tropism to all lymphoid tissues and brain tissues. In lymphoid tissues, CDV antigen was detected in all lymphoid tissues of CDV infected dogs, especially in nucleus and cytoplasm of lymphocytes, follicular dendritic cells, interdigitating cells and macrophages. The apoptotic cells were associated with the infection of CDV. The cell in lymphoid tissues was infected badly and the apoptosis was developed evidently. In brain tissue, the most severe infectious cells were cerebral blood vessel endothelia, ependymal cells, astrocytes and oligodendrocytes. Secondly it was microglias, neuron in nervenucleus. Less infectious cells were pyramidal cells and Purkinje cells. The most obvious apoptotic cells were cerebral blood vessel endothelia, astrocytes, and oligodendrocytes. Secondly it was ependymal cells and neurons in nervenucleus. As severe infection, more apoptotic cells were observed in the pyramidal cells, Purkinje cells and neurons in spinal cord. It follows that CDV has varying degree tropism to all lymphoid tissues and brain tissue. The stronger cells of tropism to CDV are, the faster and earlier apoptosis is. Apoptotic cells in lymphoid tissues and brain tissues may be related to lymphocytopenia, immunosuppression and the appearance of diverse neurological signs in clinic.

Key words: apoptosis; lymphoid tissue; brain tissue; immunosuppression; neurological sign

Introduction

Canine distemper is an anthropozoonosis and can cause human Paget's disease of bone [1,2]. At present, canine distemper is still popular all around the world and gives rise to serious jeopardizing for human public health. Canine distemper virus (CDV) is a pantropic, single-stranded RNA morbillivirus, belongs to the family paramyxoviridae [3,4] and infects different cell types, including epithelial, mesenchymal, neuroendocrine and hematopoietic cells of various organs and tissues. CDV infection of dogs is characterized by a systemic and/or nervous clinical course and viral persistence in selected organs including lymphoid tissue and the central nervous system.

It had been known [5,6] that CDV infection caused lymphopenia and immunosuppression in dogs during the early phase of disease, which is responsible for the high morbidity and mortality induced by secondary infections. Previous studies have proved that the tropism of CDV is for lymphocytes. Experimental infection of dogs with virulent CDV strains, demonstrated an initial infection of lymphoid cells in spleen, lymph nodes, MALT and thymus [7]. Pathologically, a lot of studies [8 – 11] have proved that the acute primary encephalic lesions induced by CDV are only demyelinating change and no inflammatory reaction. So the encephalic lesions should be called encephalopathy or demyelinating encephalopathy. Demyelinating encephalopathy is a most common manifestation of canine distemper in the early stage of the disease. Encephalitic lesions are generally secondary changes to the demyelination. CDV can induce disseminated and multifocal demyelination in CNS [11 – 14]. Clinically, Nervous signs are one of the most common symptoms in dogs with canine distemper, but expressional neurological symptoms of ill dogs are not the same on the clinic.

Up to now, the pathogenesis of lymphopenia and appearance of different nervous signs is still too unclear. In order to research the causes of lymphocytes depletion and why to occur different nervous signs, this experiment was performed.

Material and methods

Animals

22 dogs used in this research were divided into two

groups. The group 1 dogs (n = 16, Nos. 1 – 16, experiment group) were suffering from spontaneous acute canine distemper diagnosed by clinic with anti-CDV monoclonal antibody. The group 2 animals consisted of three males and three females healthy Beagles with age of 6 months (n = 6, Nos. 17 – 22, control group) served as control animals from a toxicological study about traditional Chinese medicine, and were vaccinated and wormed according to standard protocols.

Collection of tissue samples and sections

The lymphoid tissue samples (spleen, lymph node and Peyer's patchs) and brain were collected form animals in necropsy. The samples were fixed in 10% neutral buffered formalin. In order to detect the brain lesions in detail the fixed brain tissue was split into left and right brain tissue along longitudinal fissure, and then each part was divided into cerebrum, cerebral stem and cerebellum, respectively. The all tissues were then dehydrated by alcohol, and embedded in paraffin, sectioned at 4 μm thickness and stained with hematoxylin and eosin (HE). The sections were observed under light microscope.

Anti-CDV immunohistochimstry assay

To detect the CDV, the streptavidin-biotinlated peroxidase complex (sABC) method was used. Briefly, the deparaffinized and rehydrated sections were incubated with 3% hydrogen peroxide in methanol solution at room temperature for 10 minutes; treated by autoclave sterilizer in citrate buffe at 120℃ for 20 min; blocked with normal goat serum at room temperature for 10 min; incubated with primary antibodies (1∶256, Vector Laboratory) at 4℃ for 48 hours; secondary antibodies (biotinylated goat anti-mouse and rabbit IgG, Vector Laboratory) at room temperature for 10 minutes; incubated with sABC for 5 min at room temperature. After application of each reagent the sections were rinsed with PBS three times at room temperature for 5 min. Finally, the sections were incubated with DAB, and then lightly counterstained with hematoxylin.

TdT-mediated nick end-labeling assay (TUNEL)

To specifically detect DNA strand breaks, the terminal deoxynucleotidyl transferase (TdT)-mediated nick end-labeling (TUNEL) technique was used. The apop tag plus in situ apoptosis detection kit (DAKO EPOS) was employed in this experiment. Briefly, the sections were incubated with proteinase K at room temperature for 15 min; with 2% hydrogen peroxide in PBS, at room temperature for 5 min; with equilibration buffer at room temperature for 30 min; with TdT enzyme digoxygenin reaction buffer at 37℃ for 60 min; with stop buffer at room temperature for 10 min; and with anti-digoxygenin peroxidase at room temperature for 30 min. Finally, the sections were incubated with DAB, and then lightly counterstained.

Results

Apoptosis occurred in lymphoid tissues

CDV antigen was detected in all lymphoid tissues of CDV infected dogs, especially in nucleus and cytoplasm of lymphocytes, follicular dendritic cells, interdigitating cells and macrophages. CDV antigen was not observed in proliferating reticular cells and fibroblast. There were different changes in dissimilar lymphoid tissues. In lymph nodes distinct border was not observed between the cortex and medulla, CDV antigen was located in cortex and paracortical areas (Fig. 1A). In spleen, the strong positive reaction was found in the cells in splenic nodules, periarterial lymphatic sheaths (PALS) and splenic cord. In the region of the Peyer's patchs, the positive cells were among the cells infiltrated in lamina propria of villi and the mucosa epithelium. The apoptotic cell in lymphoid tissues was associated with cell infected by CDV. The cell was infected badly and the apoptosis was developed evidently. In lymph nodes a lot of lymphocytes with pyknosis brown nuclei expressed TUNEL positive reaction and dispersed in follicular like area, paracortical area and medullary cords. Some of follicular dendritic cells showed apoptotic positive reaction (Fig. 1B). In the spleen apoptotic lymphocytes mainly scattered in the splenic nodules, PALS and splenic cord. In the Peyer's patchs lots of TUNEL positive cells principally decentralized in aggregated nodules-liking area. A few of apoptotic lymphocytes were examined in the lamina propria of intestinal villus.

Apoptosis occurred in brain tissue cells

The results demonstrated that CDV had varying degree tropism to all brain tissue cells. The most severe infectious cells were cerebral blood vessel endothelia, astrocytes (Fig. 2A), ependymal cells and oligodendrocytes. Secondly it was microglias, neuron in nervenucleus of white matter. Less infectious cells were pyramidal cells and Purkinje cells. Apoptotic cells in brain tissues had occurred all of brain tissue cells, too. The most obvious apoptotic cells were cerebral blood vessel endothelia, astrocytes (Fig. 2B), and oligodendrocytes. Secondly it was ependymal cells and neurons in nervenucleus of white matter. As severe infection, more apoptotic cells also presented in the pyramidal cells in cerebral cortex and Purkinje cells in cerebellum. Comparing with control dogs, the GFAP in astrocytes and GalC in oligodendrocytes increased evidently in brain tissue of dogs with canine distemper. It suggested that the metabolism of astrocytes and oligodendrocytes had occurred disorder.

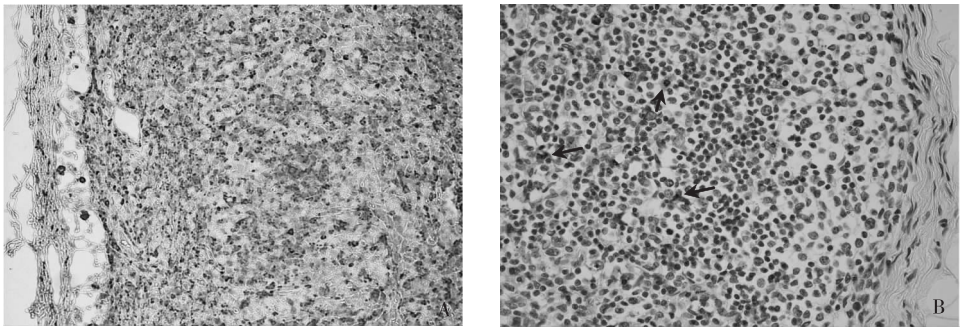

Fig. 1 Reaction of anti-CDV antigen and apoptosis in lymphoid tissue
A. Lots of positive lymphocytes and follicular dendritic cells of anti-CDV antigen in the cortex and paracortical areas of lymph node. sABC staning ×100.
B. Many apoptotic lymphocytes and follicular dendritic cells (arrows) in lymphoid tissue. TUNEL staining ×200.

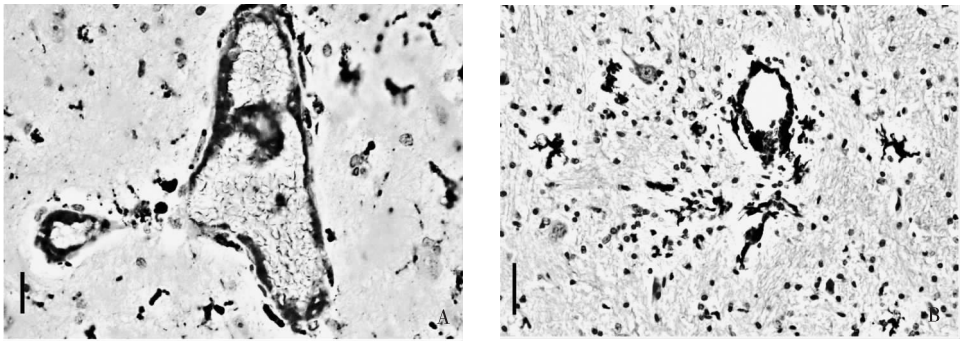

Fig. 2 Reaction of anti-CDV antigen and apoptosis in brain tissue
A. Strong positive reaction of ante-CDV antigen in vascular endothelia and astrocytes. sABC staning ×400.
B. A lot of apoptotic astrocyte and vascular endothlium in brain tissue. TUNEL staining ×200.

Discussion

Relation of lymphopenia, immunosuppression and apoptosis in lymphoid tissues

The depletion of lymphocytes, absence of secondary follicles, loss of primary follicles and formation of follicle-like areas in lymphoid tissues of dogs with CDV and association with immunosuprresion has been widely reported by numerous studies on the spontaneous or experimental cases, but the reason that caused depletion of lymphoid tissues was still too unclear. Iwatsuki et al. [15] reported that the diminished immune function in the early phase of the disease is associated with viremia and partially a consequence of lysis of lymphocytes and macrophages. Viral antigen is located in T-cell-dependent areas and in the follicles of lymphoid tissues during the acute phase. Beineke et al. [11] reported that CDV is a lymphotrophic and highly immunosuppressive infectious agent and can causes a long lasting and profound inhibition and impairment of cellular and humoral immune functions characterized by immunosuppression, lymphocyte loss, and leucopenia. Additionally, natural and experimental CDV infection causes an acute systemic, often fatal and severely immunosuppressive disease in ferrets. Leukopenia and an inhibited cellular immune response, such as suppressed delayed type hypersensitivity and a decreased lymphocyte proliferation activity as well as a reduced humoral immune response have been reported [16] in experimentally CDV-infected ferrets.

According to this study, the depletion of lymphoid tissues and immunosuppression were related to the apoptosis of lymphocytes. In the acute phase of CD, the absence of focal or diffusive necrotic lymphocyte was observed, but lots of pyknotic lymphocytes were examined in lymphoid tissues. By TUNEL assay, the pyknotic lymphocytes were proved to be a kind of apoptotic cell. Apoptotic lymphocytes showed in every kind of lymphoid tissues, such as spleen, lymph nodes and Peyer's patchs. It was testified that apoptotic lymphocytes included T cells mainly located in paracortex zone, PALS and diffusive lymphatic tissues, and B cells primarily situated lymphatic follicles, medullary cord and splenic cord. Therefore, the depletion of lymphoid tissue, lymphopenia

and immunosuppression that contained cellular and humoral immune function might be associated with a great number of apoptotic lymphocytes in the acute period of dogs with CD. In additionally, apoptosis of antigen presenting cells, such as follicular dentritic cells, interdigitating cells and macrophages was related to immunosuppression.

Relation between different neurological signs and apoptosis in brain tissue cells

The neurologic signs are often an important symptom in dogs infected with CDV. Neurologic complications of canine distemper are the most significant factors concerning prognosis and recovery from infection. Although the disease may occur in a mild and nonfatal form, most animals with severe nervous succumb to the infection. Initial nervous symptoms, characterized by chewing movements, excessive salivation, epileptiform seizures, and occasionally neuromuscular tics, are prominent in some outbreaks, having been observed in 50% of affected animals. Often neurologic signs are not manifest until after the respiratory signs have abated. Major nervous signs are diverse [17] and include myoclonus, nystagmus, ataxia, postural reaction deficits, tetraparesis, plegia and so on [14,18,19]. In the clinic the dogs with distemper often have different neurological signs that are difficulty to diagnosis [20].

Why does same disease for dogs manifest different nervous signs in the clinic? It is also too unclear now. This study demonstrated the neurologic signs of dogs with CD could be of any type, depending upon the region of the CNS that was damaged by the virus. When dog is infected by CDV, at the acute stage, virus is found in every secretion and excretion of the body. As neurological signs showed in the clinic, CDV can be found in all of brain tissue cells, especially in vascular endothelium, astrocytes, oligodendrocytes and ependymocytes. Following the CDV replication in their cytoplasm a lot of brain tissue cells developed the apoptosis, which could cause different damages of brain tissues. Apoptotic vascular endothelium and ependymal cells could make the blood brain barrier destroy and cerebrospinal fluid circulation disturb and stir up CDV easily to the brain tissues. Apoptotic astrocytes did not only cause disturbance of blood brain barrier, but also lead to the disturbance of metabolism in oligodendrocytes and neurons. Apoptotic oligodendrocytes could arouse the demyelinating encephalopathy. Apoptotic neurons in the different region of brain would give rise to the homologues nervous dysfunction and manifest diverse neurological signs in the clinic..

Conclusion

It follows that the depletion of lymphoid tissue, lymphopenia and immunosuppression that contained cellular and humoral immune function may be associated with a great number of apoptotic lymphocytes in the acute period of dogs with CD. In additionally, apoptosis of antigen presenting cells, such as follicular dentritic cells, interdigitating cells and macrophages was related to immunosuppression. Because CDV has varying degree tropism to all brain tissue cells, the stronger cells of tropism to CDV are, the faster and earlier apoptosis is. This may be involves the CDV entered to brain tissues. The appearance of diverse neurological symptoms in clinic may relate to apoptosis of neurocytes located in the different position.

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Effectiveness Evaluation Coccidiostatic Sulfadoxine/Trimethoprim and Nitazoxanide, in Dairy Goats

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Summary: Objective was the comparison of effectiveness as coccidiostat of sulfadoxine/trimethoprim and nitazoxanide for control of *Eimeria* spp., using 12 primiparous Saanen goats and 12 alpine goats at a farm located in Morelos State. Both groups was applied a medium dose for parasitic control, the first group received treatment with sulfadoxine/trimethoprim using a subcutaneous dose of 3 ml for each 50 kg of weight while the second group was treated with Nitazoxanide using an oral dose of 0.7 ml per kg of body weight. Effectiveness was calculated according to the percentage of reduction of the eggs of *Eimeria*, obtained through comparison of the samples 1 and 2 using the McMaster technique, resulting in an effectiveness of the 54.49% respect to sulfadoxine/trimethoprim and the 92.66% in the primiparous goats treated with Nitazoxanide. Nitazoxanide is more effective for these eggs compared with sulfadoxine/trimethoprim; this may be a consequence of the resistance obtained by the prolonged use of this as a treatment.

Introduction

Recent years have seen increased interest in goat's milk and its products, mainly cheese. This situation has a significant impact on the development of goat farms in the world. The overall state of the world's goat population is about 704 million individuals. The largest population has been demonstrated in Asia (over 465 million), followed by Africa (183.5 million goats), America (35.8 million) and in Europe (18 million). Mexico with 9.5 million is second place in Latin America.

Coccidiosis is one of the most economically devastating parasitic diseases of small ruminants. These infections remain among the leading causes of morbidity and mortality in the world today

Sulfonamides are the oldest and remain among the most widely used antibacterial agents in veterinary medicine, because of low cost and their relative efficacy in some common bacterial diseases. After the introduction of the nitroimidazoles and benzimidazoles, there have been few, new innovations in treating intestinal parasitic infections.

Resistance to pyrimethamine was described at the outset of its use, due to a mutation in one of the reaction steps, thus increasing the dose response improves not.

In a general way, most drugs antiprotozoal affect metabolism biosynthetic, anthelmintic while affect energy metabolism or neuromuscular function.

Nitazoxanide [2-acetyloxy-*N*-(5-nitro-2-thiazolyl) benzamide] (NTZ) is a broad-spectrum drug that is efficacious for the treatment of infections caused by amitochondriate luminal Parasites and helminthes(Gilles, 2002; Ortiz, 2002; Rossignol,2006).

Recent advances with the development of nitazoxanide (NTZ), a product developed specifically for the treatment of intestinal parasitic infections. The spectrum of activity against common, emerging and resistant intestinal protozoa, and common intestinal helminthes by NTZ offers potential for significant improvement in treatment outcomes for patients with intestinal parasitoids.

NTZ, 2-acetolyloxy-*N*-(5-nitro-2-thiazolyl) benzamide, was first described in 1984 as a human cestocidal drug which was effective in a single dose against *Taenia saginata* and *Hymenolepis nana* (Rossignol, 1984)

Development of the NTZ was re-initiated in 1994 after the anti-protozoan activity of this drug was discovered. Since 1994, there have been several reports of pre clinical and clinical studies evaluating the activity of NTZ against a broad spectrum of protozoa and helminthes that infect the intestinal tracts of humans.

Recent studies in anaerobic protozoa and bacteria (Trichomonas vaginalis, Entamoeba histolytica and Clostridium perfringens) and in the microaerophile Helicobacter pylori have shown that NTZ inhibits pyruvate ferredoxin oxidoreductase (PFOR). It has been speculated that this is due to the action of PFOR (pyruvate ferredoxin oxidoreductase or other nitro-reductases), which could reduce the nitro-group and kill anaerobic intestinal bacteria through the production of free radicals (Hemphill,2006).

Mechanism of action: 1 Blocks aerobic glucose cycle, so that the products of metabolism of glucose in

water and do not end CO_2, but ends up anaerobically, so the result is lactic acid creates a hostile environment for microorganisms, which virtually destroys.

The number of oocytes present in feces is influenced by the genetically determined reproductive potential of the species, the number of infective oocytes ingested, stage of the infection, age and immune status of the animal, prior exposure, consistency of the fecal sample, and method of examination

Once the disease has been acquired it is just possible to take medication. Studies on the way of action allow knowing more about the physiology of the parasite and, on the other hand, to understand better the physiology of the parasite allows designing new more effective drugs.

Eimeria can invade and destroy intestinal cells of the hosts, causing anemia, electrolyte loss and poor absorption of nutrients. The most common sign of infection is diarrhea, and affected goats can show a rough hair coat, poor weight gain and weakness (Wang et al., 2010).

Material and methods

Before this study was conducted another study in same farm, with 28 samples were obtained from 14 different pens. Were identified 2, specifically coccidia Eimeria crandallis and Strongyloides eggs and larval stage Haemonchus contortus. Prevalence rate, Eimeria crandallis was positive in 50% overall herd. The prevalence of positive animals to Haemonchus contortus was 28.57% of the total herd. The total prevalence of positive animals in the for Coccidia and Strongyloides was 22.68%.

This study were carried out on 12 primiparous Saanen goats and 12 alpine breed of a farm located in Morelos State. Animals are raised intensively.

Fecal samples were examined by using Floatation method and McMaster Technique as described by Soulsby (1986). Fecal oocytes count was carried out by using a modified McMaster technique. After examination of fecal samples the positive samples of *Eimeria* oocytes and other nematodes were subjected to sporulation in 2.5% Potassium dichromate. The oocytes of mixed species were allowed to sporulate at room temperature for 3 – 4 days.

12 primiparous Saanen goats received treatment with sulfadoxine/trimethoprim using a subcutaneous dose of 3 ml for each 50 kg of weight and 12 alpine breed groups was treated with Nitazoxanide using an oral dose of 0.7 ml per kg of body weight.

Was evaluated the behavior of both drugs after two weeks by counting oocytes of *Eimeria*.

Results and discussion

Effectiveness was calculated according to the percentage of reduction of the eggs of Eimeria, obtained through comparison of the samples 1 and 2 using the McMaster technique, resulting in an effectiveness of the 54.49% respect to sulfadoxine/trimethoprim and the 92.66% in the primiparous goats treated with Nitazoxanide.

According to our data we can see that the dairy goats with nitazoxanide in the treatment was effective with 92.66% compared to sulfadoxine/trimethoprim with 54.49% using a subcutaneos dose of 3 ml for each 50 kg of weight.

There was no significant variance for differences in age, sex and race, among the animals treated with Nitazoxanide.

Nitazoxanide effectiveness against strongyloides was not positive their effectiveness rates were less than 50%.

Conclusions

By contrast to the nitroimidazoles, NTZ appears to interact directly with PFOR (i.e. NTZ is not dependent on reduced ferredoxin), and the products of NTZ activation do not induce mutations in DNA. This distinct mechanism of action is important in explaining the therapeutic efficacy of this drug against organisms displaying high level of resistance to metronidazole (Abboud, 2001).

NZT is an excellent option for the control of coccidiosis goats farms since demonstrated a good response with a single dose. Nitazoxanidehad no significant effect on fertility and mutagenesisin animals. Therefore can be used in pregnant goats.

Nitazoxanide is a step forward in the treatment of gastrointestinal infections because of its efficacy against difficult to treat organisms such as Cryptosporidium, and its broad spectrum of activity, including the main types of gastrointestinal parasites. The use of this relatively inexpensive antiparasitic agent worldwide can be helpful in reducing the impact of gastrointestinal infections by protozoa and helminthes,

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Comparative Efficacy of Various Anthelmintics against *Haemonchus contortus* in Goats

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Summary: An experimental study to investigate the susceptibility of *Haemonchus contortus* to commonly used anthelmintics (Valbazen, Levamisole and Dectomax) was conducted using faecal egg count reduction test (FECRT) and egg hatch assay (EHA). Thirty two goats (4 groups: 3 treatments A, B, and C versus control-D, n = 8 per group) with naturally acquired pure *Haemonchus contortus* infection were used for this study. The first group (A) was treated orally with Valbazen at dose rate of 1 ml/20 kg body weight. The second (B) and the third group (C) were treated with Levamisole and Dectomax at the dose rate of 5 ml/15 kg & 1 ml/33 kg body weight respectively while the fourth group(Control) was left untreated. The overall mean percent of faecal egg counts on day 10th and 14th post treatment showed significant decrease ($P < 0.05$) in fecal egg count. The higher reduction percentage (91.8%) of faecal egg count (FEC) was recorded with Dectomax followed by Valbazen (88.6%) and Levamisole (83.4%) on day 14th post treatment suggesting dectomax as the most effective of the three anthelmintics tested in this study.

The Benzimidazole (pure) was assessed for egg hatch inhibition ability against *Haemonchus contortus* eggs and lethal dose (LD_{50}) was calculated graphically from linear regression. The eggs (200/1ml) were incubated with different concentrations (i. e., 3.0, 1.5, 0.75, 0.375, 0.1875, 0.0937, 0.0468, 0.0234, 0.0117 and 0.0058 μg/ml) at 22℃ for 48hrs. Egg hatching inhibition at different concentrations ranged from 3.5 to 81.2% and LD_{50} was 0.1202 μg/ml which is higher than the recommended concentration of 0.1 μg/ml. This indicates the presence of the resistance in eggs to Benzimidazole.

Key words: anthemintics, *Haemonchus contortus*, faecal egg count reduction test (FECRT), egg hatch assay (EHA), LD_{50}

Introduction

In Pakistan, goats are predominantly reared by the small and landless farmers who drive their livelihood by selling milk or for meat purpose. Pakistan possesses an estimate of 61.4 million goats, which produce 0.76 million tons of milk and over 0.28 million tons of mutton. In addition, goats provide hair, leather and manure (FAO, Statistics division, 2011).

Helminthiasis cause mortality, reduction in weight gain, and loss of production in small ruminants worldwide (Ketzis et al., 2002; Diehl et al., 2004; Iqbal and Jabbar, 2005; Chaudhary et al., 2007). Haemonchosis is the primary problem of nematode infection of domestic animals and frequent in sheep & goats worldwide (Nwosu et al. 2007; Tariq et al. 2008). Helminthes control in domestic animals has been achieved almost exclusively by pharmaceutically derived anthelmintics. Indeed, synthetic and semi-synthetically produced anthelmintics have for long been considered, the only effective method of controlling helminthosis. The misuse and or widespread intensive use of sometimes poor quality synthetic or semi-synthetic anthelmintics has led to development of high level multiple anthelmintic resistance that may cause failure of control of worm parasites in farm animals. (Githiori, 2004; Bizimenyera et al., 2006). In Sindh (Pakistan), there are a few studies regarding efficacy and resistance against the common dewormers being used in the field as prophylactic and therapeutic agents. Hence, the present study was designed to determine to evaluate the efficacy of various anthelmentics under local conditions.

Material and methods

The field trials were conducted at Livestock farm, Sindh Agriculture University, Tandojam, and small farmer goat farms at Tadnojam & surrounding areas, Sindh, Pakistan.

Faecal egg count reduction test

For the FECRT, 32 goats of either sex, 06 months to +1 year old (4 groups: A, B, and C versus control-D, n = 8 per group) with naturally acquired pure *Haemonchus contortus* infection were used for this study. The first group (A) was treated orally with Valbazen at dose rate of 1 ml/20 kg body weight. The second (B) and the third group (C) were treated with Levamisole and Dectomax at the dose rate of 5 ml/15 kg & 1 ml/33 kg body weight respectively while the fourth group (Control) was left untreated. The goats were marked by ear tags. The faecal samples were collected & examined for the establishment of pre-treatment eggs per gram (EPG) counts by following the method of Whitlock,

(1948), and Urquhart, et. al., (1996) worm egg counting "method for quantitation of eggs per gram (EPG)". Faecal samples were collected from each animal at 0 day (pre-treatment) and at 3rd, 7th, 10th and 14th day (post-treatment).

The eggs were identified by the culture of positive faecal samples, recovery and identification of L_3 Larvae of different species of GIT nematodes were carried out according to the techniques described by Baermann technique (1917), and Dikmans and Andrews (1933) respectively. The estimation of anthelmintic efficacy was carried out according the field controlled faecal egg count reduction test(Coles et al., 1992; Taylor et al., 2002; Coles et al., 2006). The percentage of FECRT was calculated using the following formula:

$$\%\,\text{FECR} = a - b/a \times 100.$$

Where, a = EPG pre-treatment, and b = EPG post treatment.

An AR against an anthelmintic drug is considered present if the reduction after treatment is lower than 95% and the lower 95% confidence limit is below 90% (Coles, et al., 1992)

***In vitro* egg hatch assay (EHA)**

The Egg Hatch Assay (EHA) was employed as described by Murphy, 1993. One ml of BZ serial dilutions (0.0058 – 3.0 μg/ml) dissolved in dimethyle sulfoxide (DMSO) were placed into each well of 24-well flat bottom microtitration plate. Eggs (200 /0.1 ml distilled water) were added to 1 ml BZ solutions and the control well and were incubated at 22℃ for 48 hours. The plates were kept in sealed plastic bags with water to prevent evaporation. A drop of dilute Lugol, s iodine solution was added to each well for termination of the experiment. Unhatched eggs and first stage larvae were counted in each well under ×40 magnification in duplicate and percent hatch was calculated.

Statistical analysis:

Results of faecal egg count reductions (FECR) were expressed as mean ± SD, and differences among intervals were analyzed trough ANOVA followed by Tukey's test. Statistical significance was set at $P < 0.05$ bimarginally. GraphPad Instat v3.05 was used to perform statistical analysis.

The data from Egg Hatch Assay (EHA) were transformed by Probit transformation and plotted against the logarithm of concentration. Probit transformation was performed to transform a typical sigmoid dose-response curve to linear function. The effective dose (LD_{50}) values for Benzimidazole were calculated graphically from linear regression. A log dose probit line for egg mortality was derived from the data and LD_{50} (log concentration of drug that prevents 50% of the eggs from hatching) was derived from the graph or was calculated by Probit analysis (Finney, 1971).

Results and discussion

The results of FECRT after treatment of three commonly used commercial drugs i. e., Valbazen, Levamisole and Dectomax as per experimental design are given in Table 1. The results evealed a gradual decrease in faecal eggs counts (FEC) and were significant ($P < 0.05$, $P < 0.01$) on 10th and 14th day post treatment from the 0 day pre-treatment. The administration of Valbazen decreased the FECs from 4787.5 to 1425 and 543.8 on 10th and 14th days i, e., 70.2 and 88.6% respectively. Similarly, the FECs decreased from 4512.5 to 2125 (52.9%) & 750 (83.4%) in Levamisole and from 4425.0 to 931.5 (79.0%) & 362.5 (91.8%) in Dectomax on 10th and 14th days respectively. The findings are not in agreement with the observations of keyyu, et al. (2002), waruiru (2002), Munyua et al. (2004), Ram, et al. (2007), Godara, et al. (2011) who have reported 97%, 100%, >96%, 96% and 94%, with Levamisole & Ivermactin, and 63.70% & 98.11% reduction in FECs on day 14th post treatment. The study indicated that Valbazen, Levamisole and Dectomax at the recommended dosage were not effective against *H. contortus* in goat. Reduced efficacies of anthelmintics against GIT nematodes in goats have been documented earlier by several workers Yadav and Uppal (1992), Singh et al. (2002), Waruiru et al. (2003), Ram et al, (2007)

The results of the EHA and the corresponding LD_{50} are presented in Table 2. The higher concentrations showed higher inhibition rate as compared to lower concentrations and control. In general, the egg hatching ranged from 3.5% to 81.2% at different concentrations in *H. contortus* infection and LD_{50} in this experiment was 120.22 ng/ml (0.12022 μg/mL) concentration of benzimidazole which is higher than 0.1 μg/ml. This result is not in line with the findings of Varady et al. (2007) and Sileshi et al. (2012). The result of LD_{50} is also not in line with WAAVP guideline of susceptibility range ($LD_{50} < 0.1$ μg/ml). This indicates the presence of the resistance in eggs to Benzimidazole.

Conclusions

The results of this study show that Valbazen, Levamisole and Dectomax at the rcommended dosage were not effective against *H. contortus* infection in goats under local conditions. In Egg Hatch Assay, dose dependent egg hatch inhibition was observed and corresponding LD_{50} was 0.12022 μg/ml, which is higher than 0.1 μg/ml. This indicates the presence of the resistance in eggs to Benzimidazole.

Table 1 Mean ± SD of eggs per gram (EPG) and reduction percentage of *Heamonchus contortus* (pure) in goats before and after treatment

Groups	No. of goats (n = value)	Drugs used	Pre-treatment	Post-treatment			
			0 day	3rd day	7th day	10th day	14th day
A	8	Valbazen	4787.5	3825 ± 6.5(20.1)	2312.5 ± 10.4(51.7)^{ns}	1425 ± 8.3(70.2)*	543.75 ± 5.7(88.6)**
B	8	Levamisole	4512.5	3856.3 ± 6.2(14.5)	3175 ± 8.3(29.6)	2125 ± 1.8(52.9)	750 ± 8.3(83.4)**
C	8	Dectomax	4425.0	3631.3 ± 0.9(17.9)	2381.3 ± 8.7(46.2)	931.3 ± 5.5(79.0)**	362.5 ± 4.3(91.8)**
D	8	Control	3962.5	3800 ± 13(4.1)	3912.5 ± 28.3 (1.3)	4137.5 ± 56.2(−4.4)	4262.5 ± 78.3(−7.6)

ns = P > 0.05, * = P < 0.05, * * = P < 0.01

Table 2 Percent eggs hatched at different concentrations of Benzimidazole in *H. contortus*

S. No	BZ(μg/mL)	BZ((ng/mL)	Hatching (%)	Log (BZ)	Probit (hatching)
1	0.0058	5.8	81.2	0.763428	5.88
2	0.0117	11.7	77.0	1.068186	5.74
3	0.0234	23.4	70.2	1.369216	5.52
4	0.0468	46.8	65.2	1.670246	5.38
5	0.0937	93.7	57.5	1.971740	5.18
6	0.1875	187.5	51.0	2.273001	5.03
7	0.375	375	43.2	2.574031	4.82
8	0.75	750	38.0	2.875061	4.70
9	1.5	1500	23.7	3.176091	4.26
10	3.0	3000	3.5	3.477121	3.12

Log LD_{50} = 2.0771, LD_{50} = 120.22 ng/ml

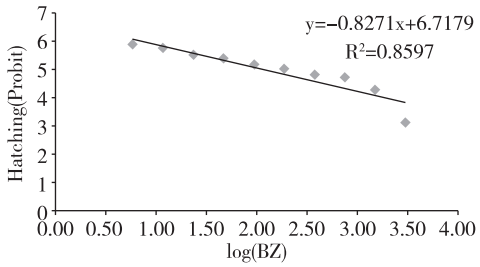

Fig. 1 Log-dose probit response line of Benzimidazole for *H. contortus* infection

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Evaluate the Effect of Pad Cooling System in Laying Hens Barn in Southeast China using CFD Simulation

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Summary: Microenvironment around hens is critical to them during hot and humid summer, when temperature is over 35℃ and relative humidity is higher than 80%. Most of the year, a microenvironment within the thermoneutral zone is provided by using mechanical ventilation and evaporative cooling systems. However heterogeneity of temperature within animal barn can be critical during hot and humid periods, particularly in high rise barns for laying hens. Therefore it is still necessary to evaluate the efficiency of current ventilation and cooling system. It is the basic of the following design improvement of ventilation and cooling for these houses. Computational Fluid Dynamics (CFD), associated with limited field measurements, can overcome the weakness of uncontrollable weather conditions and limited number of sampling points.

The objective of this study was to evaluate the ventilation of a typical laying hen house with evaporating cooling systems with respect to temperature in hot and humid regions. First, a field experiment was conducted in a typical laying hen barn (15000 hen high rise) located in the southeast of China. It included 16 positions of airflow velocity, temperature and relative humidity inside and one position outside the barn. The results of CFD simulation for the velocity and temperature were in agreement with observed data. According to the simulation result, the condition inside barn could not match the requirement of hens in a hot humid condition. The distribution of both temperature and air velocity were not uniform enough for laying hen living and production, especially in the middle part and the fans side part, the environment was much worse than the beginning of ventilation.

Introduction

An adequate environment factor such as temperature, humidity, ventilating rate, ammonia, and dust within poultry houses is a very important requirement for success in the poultry industry [1]. Truly, the environment poultry lived in is crucial to their growing especially when the gene is good. Therefore, well environmental conditions are the powerful guarantee of the hereditary basis and nutrition to achieve the best level. High ambient temperatures can have a major impact on the performances of commercial poultry. When they are coupled with high humidity, the combination can become critical. A hot and humid condition will increases the likelihood of heat stress in hot weather. Consequently, in the hot wet summer, an adequate heat and moisture removed by the ventilation system is the crucial point, to optimize the indoor climate to increase poultry performance. On the other hand, airflow and temperature distribution impact on the heat stress loss in summer and the healthy environments in animal houses.

Currently, using the evaporative pad cooling system in poultry house in summer is common and suitable in many regions in China while considering the reduction of indoor temperature[2]. However, whether the combined physical condition of temperature-humidity-velocity in animal building with pad cooling is suitable for poultry or not is not clear as well as the distribution of indoor air flow and temperature. Therefore it is imperative to evaluate the performance of pad cooling system from air flow and temperature distribution. Wang 2008 conducted field experiments, and air temperature, relative humidity, gas concentration and other parameters for pad cooling laying hen house. However these studies were performed using limited measurement points and the data obtained could not indicate the details of every location in the house. Recently, the aerodynamic methods such as computational fluid dynamics (CFD) have been used to overcome these limitations. It has great potential to predict these parameters without the drawbacks of field measurements [3]. With the help of CFD simulation result, the indoor micro-environment can be analyzed and evaluated much more accurately using CFD[4 -5].

The simulation result was agreed with the actual condition, where the amendatory K-ε model was adopted to calculate air flow field of a mechanical forced ventilated livestock building (Hoff, 1992), and the distribution of the air flow field of a high-rise laying hen house was simulated by applying a simplified CFD model [6].

The objective of this study is to find out the drawbacks of the pad cooling system in laying hen house in the southeast of china in summer time. A field experiment was conducted at a commercial laying hen house to obtain data which were used to evaluate the simulation result. The distribution of airflow and air

temperature was quantitatively analyzed using the simulation software named FLUENT. In this paper, the results of CFD simulations are presented and discussed compared with monitoring data.

Materials and methods

The experimental laying hen house

Field experiments were conducted at a typical commercial caged-hen layer house, which was a half-year old around 15,000-hen high-rise house located in the southeast of China.

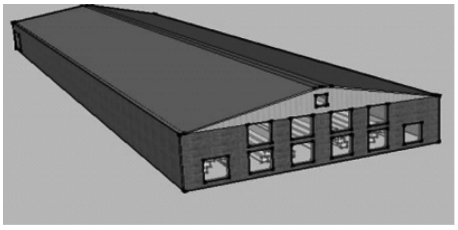

Fig. 1 Laying hen house modeling

As shown in Fig. 1, the poultry house was orientated in east-west direction and the dimensions of this building were 15 m width, 55 m length, 3.2 m eave height and 4.7 m ridge height. The foundations and plastered walls were 240 mm thick and made from concrete. The roof was covered with 1 mm thick color plate and 7 mm thick aluminum coated polystyrene foam. The house had four 50.76 m length rows of cages. Manure was scraped twice a day (8:00 am and 4:30 pm) from a 300 mm deep manure storage pit.

To reduce air temperature and improve the environment quality, pad cooling system contained evaporative pads and exhausted fans were fitted for the hot high humidweather. As shown in Fig.2, 150 mm thick pads (TaizhouLuqiaoLvjia Environment Protection Company, Taizhou, China) made from cellulose were installed at 0.2 m height from floor in the west wall and side walls closed to the west wall. These evaporative pads were cubic in shape with the same dimensions of 150 mm thick, 2 m height and the different lengths 13.5 m, 3.2 m, 1.6 m, respectively, according to the wall they located in. The pads were wetted from the pipes above them. While 10 belted exhaust fans, each 1.25 m diameter with maximum air capacities of 37,600 $m^3 \cdot h^{-1}$ (Model LJ125, Lvjiayuan, Taizhou, China) were distributed in the east wall at 30 cm height from floor, and a 750 mm diameter fan with maximum air capacities of 10,739 $m^3 \cdot h^{-1}$ located in the gable. The cooling system was controlled manually.

Fig. 2 Evaporative pads

Field measurements

The experiment was carried out under ventilation condition which is commonly used when peak temperatures are achieved during summer on the farm. The experiment was conducted to assess the indoor environmental conditions as well as provide a validation for CFD simulation. The measured data were also used to set the boundary conditions and some input values in the simulation.

As shown in Fig. 3, the internal air velocity (V) was measured at 10 points by portable three-cup anemometer (FYF-1 portable three-cup anemometer, Shanghai Fengyun Meteorological Instrument Corp. Shanghai, China). Air temperature and relative humidity were measured at 16 points shown in Fig. 3 using hygrothermograph (ZDW-Y20, Zeda Company, Hangzhou, China). All points expect point 8 and point 10 were installed at the height between 2nd floor and 3rd floor. Point 8, 10 were at different height, between 3rd and 4th floor, between 1st floor and 2nd floor, respectively. The temperatures of the internal surface of four walls were measured every 1 m by a portable high performance infrared thermometer (Raytek MX4, Fluke Company, USA), so it was the temperature of the internal surface of the roof. The field measurements were conducted at July 16th, 2012.

Computational fluid dynamic techniques

The CFD numerically solves the Reynolds-averaged form of the Navier-stokes equations (Launder and Spalding, 1974) within each cell in the domain. FLUENT software (version 6.3, Fluent Inc., NH, USA) was used as solver to calculate the nonlinear partial differential equations derived from the conservation of mass Eqs. (1), momentum Eqs. (2) and energy Eqs. (3) equations which were listed below.

$$\frac{\partial \rho}{\partial t} + \nabla(\rho \vec{v}) = S_m \quad (1)$$

$$\frac{\partial}{\partial t}(\rho \vec{v}) + \nabla(\rho \vec{v}\vec{v}) = -\nabla P + \nabla(\bar{\tau}) + \rho \vec{g} + \vec{F} \quad (2)$$

$$\frac{\partial}{\partial t}(\rho E) + \nabla((\rho E + P)) = \nabla(k_{eff} \nabla T - \sum_j h_j \vec{J}_J + (\bar{\bar{\tau}} \vec{v})) + S_h \quad (3)$$

Where S_m is mass source, $kg \cdot m^{-3}$; ρ is density, $kg \cdot m^{-3}$; u, v, w are velocity, $m \cdot s^{-1}$; t is time, s; P is pressure, Pa; τ is the stress tensor, Pa; F is external

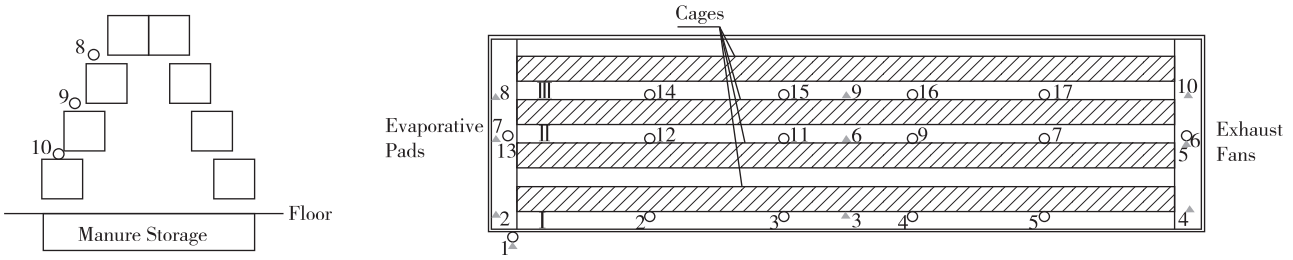

Fig. 3 Locations of measurement points

force vector, $N \cdot m^{-3}$; g is gravitational acceleration, $m \cdot s^{-2}$; E is total energy, J; k_{eff} is the heat transmission coefficient; h is specific enthalpy, $J \cdot kg^{-1}$; J is the component of diffusion flux, $kg \cdot m^{-2} \cdot s^{-1}$; S_h is total entropy, $J \cdot K^{-1}$; and T is air temperature for the livestock building, ℃.

In this study, CFD airflows were computed using Renormalization-group (RNG) k-ε turbulent model and the results were validated with the field measure data. The RNG model is less dissipative than the standard k-ε turbulent model as presented by Analytis (2003)[7]. Lee et al. (2007)[8] conducted an experiment in which the simulation results using RNG k-ε turbulent model was compared with the PIV results and concluded a-6.2% error happened in the simulation using RNG k-ε turbulent model. The RNG k-ε turbulent model is defined by the following Eqs. (4) and (5).

$$\rho \frac{dk}{dt} = \frac{\partial}{\partial x_i}\left[\alpha_k \mu_{eff} \frac{\partial k}{\partial x_i}\right] + G_K + G_b - \rho\varepsilon - Y_M \quad (4)$$

$$\rho \frac{d\varepsilon}{dt} = \frac{\partial}{\partial x_i}\left[\alpha_\varepsilon \mu_{eff} \frac{\partial \varepsilon}{\partial x_i}\right] + C_{1\varepsilon} \frac{\varepsilon}{k} (G_k + C_{3\varepsilon} C_b) - C_{2\varepsilon} \rho \frac{\varepsilon^2}{k} - R \quad (5)$$

Where k is turbulent kinetic energy, $m^2 \cdot s^{-2}$; μ_{eff} is effective viscosity ($\mu = \mu_t$), $m^2 \cdot s$; μ is viscosity, $m^2 \cdot s$; μ_t is turbulent viscosity, $m^2 s$; α_k is the generation of kinetic energy due to the mean velocity gradients, $kg \cdot m^{-1} \cdot s^{-2}$; α_ε is the generation of kinetic energy due to buoyancy, $kg \cdot m^{-1} \cdot s^{-2}$; G_k is the generation of turbulent kinetic energy due to the mean velocity gradients, $kg \cdot m^{-1} \cdot s^{-2}$; G_b is the generation of kinetic energy due to the buoyancy, $kg \cdot m^{-1} \cdot s^{-2}$; ε is turbulent dissipation rate, $m^2 \cdot s^{-3}$; Y_M is the contribution of the fluctuating dilatation in compressible turbulence to the overall dissipation rate, $kg \cdot m^{-1} \cdot s^{-2}$; $C_{1\varepsilon}$ and $C_{2\varepsilon}$ are constants of 1.42 and 1.68; $C_{3\varepsilon}$ is tanh (u_1/u_2), u_1 and u_2 are components of the flow velocities parallel and perpendicular, respectively, to the gravitational vector, and R is the gas-law constant, 8.31447 × 103 $J \cdot kg \cdot mol^{-1} \cdot K^{-1}$.

The turbulence kinetic energy (k) and turbulence dissipation rate (ε), which are important factors for inlet conditions, were calculated using Eqs. (6) and (7) [9].

$$k = \frac{1}{2}(u^2 + v^2 + w^2) \quad (6)$$

$$\varepsilon = \frac{C_\mu^{3/4} \times k^{3/2}}{1}, 1 = \min(k \times z_n, k \times \delta) \quad (7)$$

Where C_μ is an experimental constant; Z_n is height from the ground, m and δ is thickness of the turbulent boundary layer, m.

CFD simulation

Modeling set-up and general settings:

A three-dimensional simulation of the indoor airflow and temperature was carried out by the commercial code Fluent 6.3 (Fluent Inc., Lebanon, NH, USA). The geometry model similar to the reality house was built using Solidworks 2012 software (Solidworks, USA). Due to simplify the geometry model, all the cages were used 32 cubes with 37 cm width, 32 cm height and 50.7 m length to replace (Fig. 4). The internal of poultry house was considered as the three-dimensional computational domain. Three-dimensional grids in the three-dimensional computational domain were generated by GridgenV16software (Pointwise, USA). In this work, fine and dense structure meshes were selected, for the finer and denser the grids were, the more accurate the result we get. However, too fine and dense meshes needed a long time and required a super computer to calculate the governing equations.

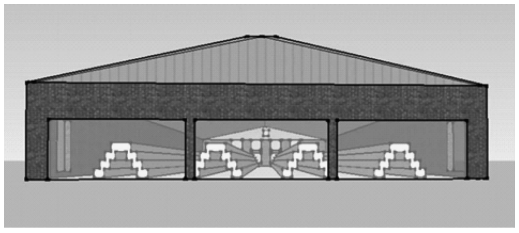

Fig. 4 The simplified modeling of the 3D structure

Boundary conditions:

Accurate use of CFD techniques involves defining correct boundary conditions. However, quantifying and

determining the boundary conditions in the laying hen house boundaries can be very difficult in practice.

In this work, the wall and floor temperatures were measured and used as the boundary condition. Temperature and velocity of the air passed though evaporative pads were measured and used to set the inlet boundary condition.

The total heat produced by laying hens was determined usingEqs. (8)[10]

$$THP = 3.2\ m \tag{8}$$

Where THP is total heat production, $W \cdot m^{-2}$;

Some boundary conditions settings were present in the Table 1.

Table 1 Input data used for Fluent

Constitution	Material	Thermal conductivity ($W \cdot m^{-1} \cdot ℃^{-1}$)	Specific heat $kJ \cdot kg^{-1} \cdot ℃^{-1}$	Density $kg \cdot m^{-3}$
Roof	Expanded polystyrene	0.029	1.254	38
Floor	Sand	1.51	1.672	1860
Wall	Air brick	0.81	887.8	1850

Simulation validation

Validation is a most significant part in CFD simulation to ensure that the CFD model conforms to real conditions[11]. Two means of validation were applied in this work, one was the error between the simulated and measured data (Ev) Eqs. (9).

$$E_v = \frac{c_p - c_m}{c_m} \tag{9}$$

Where c_p is the predicted values in simulation result; c_m is the initial data from field measurement.

Results and discussion

Field measurements

The measured data of air velocity and temperature at the inlets, outlets, and interior walls are shown in Table 2. These measurement data were used for boundary condition setting in the numerical simulation.

Table 2 Surface temperature of envelope

Surface temperature	Value (℃)	Surface temperature	Value (℃)
North wall	31	Wet pad side wall	27
South wall	32	Fan side wall	31.5

Regarding indoor measurements, air velocities measured at 9 points inside the house are shown in Fig. 3. The measurement result was shown in the Table 3(a). With all the fans running, the maximum air velocity was 2.3 $m \cdot s^{-1}$, it was measured closed to middle down fan. The maximum inlet air velocity was 1.2 $m \cdot s^{-1}$, which was obtained closed to the middle pad in west wall. The range of air velocity was from 0.6 to 2.3 $m \cdot s^{-1}$. As shown in Table 3(a), the lowest velocity was measured in the middle of the house. According to the measurement data, the air velocity is way lower than the recommended value, an air speed of 2.5 – 3 $m \cdot s^{-1}$ for layers is recommended when birds are reared under very hot conditions. Loss of air movement over the birds can result in severe heat stress[12].

As shown in Table 3(b), with all the fans running, the air temperatures measured in the laying hen house rose from 27.1 to 32℃ from pad side to fan side while the external air temperature was measured 35℃. It shows that all of the temperatures measured 16 monitoring points were much higher than the comfortable temperatures recommended[13]. Also, the relative humidity (RH) was measured and presented in Table 3(b). The values were declined from 86.0% to 71.4% from pads to fans which the mean RH was 76.2%, overpassed the recommended value 60% – 70% [14]. Laying hens raised in those high temperature and humidity combination condition were suffered heat stress.

Table 3(a) The measurement result of velocity

Measure point	Velocity value ($m \cdot s^{-1}$)	Measure point	Velocity value ($m \cdot s^{-1}$)
1	0.9	6	0.7
2	1.1	7	1.2
3	0.6	8	1.2
4	1.1	9	0.6
5	2	10	2.3

Table 3(b) The measurement result of temperature

Position	T(℃)	RH(%)	Position	T(℃)	RH(%)
1	35	49.5	10	29.8	78.8
2	29.9	77.4	11	30.3	73.5
3	30.9	71.9	12	29.7	78.5
4	30.6	74.3	13	27.1	86
5	30.8	73.2	14	29.6	78.2
6	32	72.3	15	29.7	77.3
7	31.1	74	16	31.1	76.5
8	30.9	71.4	17	30.5	77.5
9	30.0	78.6			

Analysis of the simulation results

Velocity field:

The velocity distribute along Z direction (from floor

to roof) were displayed in Fig. 5, Z = 0.5 m, Z = 0.9 m, Z = 1.3 m were chosen. It is clearly that the velocity closed to the south wall was smaller than the velocity closed to the north wall. Two factors made the contributions to this asymmetric phenomenon, that is, the difference of each wall surface temperature and the wet pad area. The air come in the house through the wet pad (inlet) at 1.2 $m \cdot s^{-1}$, whilethe velocity became lower and lower as the distance to the wet pad increasing. Especially in the middle of the barn, the velocity is only 0.5 $m \cdot s^{-1}$. However, because of the exhaust fans, the velocity closed to fans changed very quickly from 0.5 $m \cdot s^{-1}$ to 2.5 $m \cdot s^{-1}$ even much bigger. The trend of the air velocity near fan was the closer the bigger. From the contours, the biggest velocity closed cages were not satisfied the requirement of the laying hens, especially the zone under cages and closed the cages. Because of the low velocity, the heat layer produced could not be carried with the air movement and went outside.

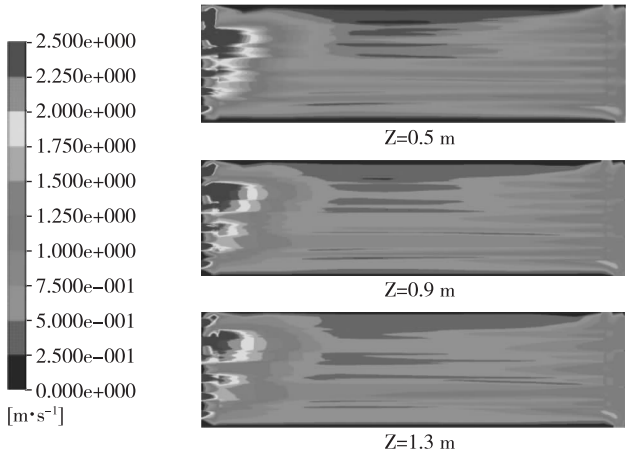

Fig. 5 The distribution of air flow alone Z direction

At the plane Z = 0.5 m, a big stagnant zone were appeared. In such zone, high temperature and high humidity would occur, and it would contribute to some bad consequences such as bad performance, even death, for the heat produced by the laying hens cannot be removed with the air flow. So this kind of situation was dangerous and fatal to the layers. In the end of the ventilation (closed to the exhaust fans), the velocity is bigger in the south side than north side. In generally speaking, all of velocities in the barn were lower than recommended.

Temperature field:

The distribution of temperature was simulated and the contours chart of Z = 0.5 m, Z = 0.9 m, Z = 1.3 m, which can provide a clear temperature field for analysis, were presented in Fig. 6.

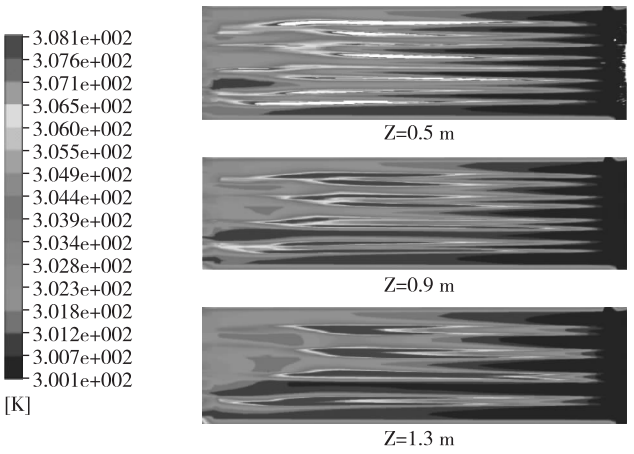

Fig. 6 The contours of temperature distribution in vertical plane of X-Y section

It shows that the temperature increased gradually from inlets to outlets. The difference of temperature was 5℃, ranged from 27℃ to 32℃. Temperature under and close to the cages increased faster than the other places because of the air flow, especially in the zone away from the exhausting fans about 10 m to 20 m. In the zone where the cages closed to the fans, because of the exhausting fans'function, the temperature was lower than the former zone.

Aiming to analysis the distribution of temperature vertically, four contoursoftemperature distribution in X direction was displayed in Fig. 7, namely Z = 0.4 m, Z = 0.8 m, Z = 1.3 m, Z = 1.7 m. The air temperature closed cages was much higher than in the aisle. Along with the distance to inlet increasing, the air temperature above the cage zones which were regarded as the laying hens' occupation rose gradually. Like the distribution of air velocity, temperature also presented an asymmetry. Temperatures closed to the south wall (right part in the pictures) were higher than the air closed to the north wall, because the radiation of sun made the difference of interior surface temperature of each wall. The less of air flow in the right part are the direct factor produced the asymmetric distribution. The temperatures closed to the cages were also rising from inlet to outlet, because of the heat increasing.

According to the simulation analysis, there were some ventilation problems through the house. Air velocity around the cages was lower than that of aisle, and the air velocity along the two sidewalls was lower than that of center. Correspondingly, temperature around the laying hen cages were higher than that of aisle, temperature at the two middle cages was higher than the bottom and top cages, and there was more than 4℃ temperature difference between the air inlet and exhausting. So the

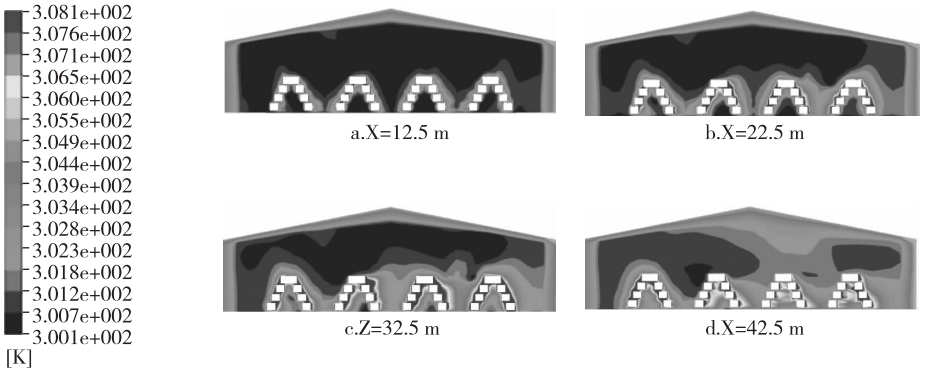

Fig. 7 The distribution of Y-Z section at different heights

laying hens were suffered from serious heat stress under this uniform air distribution condition combined with high temperature and humidity. It indicated that evaporating cooling systems without adequate air moving over the birds was ineffective in the southeast China with hot humid summer. Modifying of the evaporating cooling systems, insulation design, and indoor environment control strategies to improve the microclimate for poultry need be considered in the future.

CFD validation

The validation was carried out in order to judge the performance of the simulation results by comparing the predicted values with the field measurement data. The error between the predicted and measured data was calculated using Eqs. 9. It is shown in the result that the maximum error of air temperature and velocity were 0.055 and 0.3, respectively, the minimum error of air temperature and velocity were −0.047 and −0.467, respectively, which indicated that the simulation model was reasonable to estimate ventilation efficiency and environment conditions in the pad cooling system laying hens house.

Conclusions

Air velocities and temperatures simulations were carried out in a mechanically ventilated commercial poultry house with wet pad cooling system by means of CFD technique. The different comparisons carried out between the measured and simulated air velocities showed that the simulated air velocities could be considered a reasonable estimation of velocities in a commercial poultry house. However, an optimal and effective simplified method of envelope structure and simulation modeling is needed in the further study for a much more accurate simulation results.

The evaporative cooling and ventilation system design, and the way it was operated in the high-raise laying hens house in summer, was concluded as a method which was not appropriate for poultry farms located in hot humid climates in this study, as it did not provide high and uniform air velocity at the zone of the laying hens which was necessary to relieve bird heat stress. A better ventilation strategy under conditions of heat stress should be considered.

In conclusion, CFD simulations, when validated by experimental research, can provide useful information about the actual airflow in commercial poultry house and offer a whole set of data vertically and perpendicularly which direct measurement cannot complete. This work makes a trial toward evaluating the practical feasibility of using the CFD technique as a tool for systematically predicting air flow around the laying hen cages in commercial poultry houses.

Acknowledgement

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Introduction of Petri Net Knowledge Representation to Diagnosis Expert System of Animal Disease

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Summary: Animal disease diagnosis expert systems, which have attracted much interest of researchers, provide farmers, vets etc. with an automatic tool for diagnosis of animal disease, and can also help to manage medical records, spread veterinary knowledge and play a role in computer aided tutorial. This paper introduces a Petri nets knowledge representation tool to animal disease diagnosis expert systems. Petri nets knowledge representation method has been researched widely by many knowledge representation researchers. However, it is a novel method in the animal disease diagnosis field. The concept of Petri nets is introduced and a simple method for knowledge representation is presented as well as reasoning algorithm. The advantages of Petri nets knowledge representation are discussed and an illustration is given to show how it works.

Introduction

As one of the hottest branches of Artificial Intelligence, Expert Systems (also known as knowledge-based system, KBS) have been applied to many areas like industry, agriculture, medical treatment and so on and produced many social and economical benefits. Diagnosis expert systems, diagnosing a disease or some malfunction, are very common among these applications, for example, MYCIN-a very famous expert system helping doctors diagnosing bacteria-infected diseases developed by Stanford University [1]. In the veterinary field, with the computer-aided trend, animal disease diagnosis expert systems have been researched, designed and established [2-4].

An expert system usually consists of knowledge base, inference engine, data base, interface and so on. Knowledge acquisition, knowledge representation and reasoning algorithm are critical techniques for a successful expert system. As far as knowledge representation is concerned, there are several common-used methods such as predicate logic, production rule, fuzzy production rule, frames, semantic network, conceptual graphs, Petri nets, etc. Every knowledge representation method has its advantages and disadvantages, and most of them can be transformed into others.

Since its birth, Petri nets have been used for modeling and analyzing different systems that are characterized as being concurrent, asynchronous, distributed, parallel, nondeterministic, and/or stochastic [5]. And later on, Petri nets were used for knowledge representation[6,7]. Many researchers see Petri net as a promising tool for knowledge representation and many papers about Petri nets knowledge representation and related reasoning algorithm have been published.

Since fuzzy production rule is the main knowledge representation method concerning animal disease expert system currently and Petri nets is still untouched in this field, this paper introduces Petri nets to the knowledge representation of animal disease expert systems so as to provide an efficient tool to the organizing of knowledge base, avoid shortcomings of other methods and make a more efficient animal disease diagnosis expert system come true.

Knowledge representation

Most symptoms and knowledge with regard to animal disease are fuzzy, incomplete, and imprecise, such as that a symptom may indicate not only a disease but several possible diseases and a disease may appear some symptoms at the same time. There is a vague relationship between symptoms and diseases.

Some knowledge representation methods have been developed to deal with vague knowledge, and among them a simple and common method is fuzzy production rule [8]. Usually, a fuzzy production rule adds a CF (Certainty Factor), which is between zero to one, to the production rule so as to express fuzzy relationship between two propositions-the antecedent and consequence. The general form of a fuzzy production rule is as follows:

$$R_i: \text{IF } d_j \text{ THEN } d_k (CF = \mu_i)$$

Where d_j is the antecedent of the rule, d_k is the consequence of the rule. (Both d_j and d_k are propositions which may contain fuzzy terms and can be judged by the degree of truth.) μ_i is the certainty factor of the rule. And whether the rule can be fired is determined by the degree of truth of antecedent d_j (θ_j) compared with the threshold: if the degree of truth is greater than the threshold, the rule can be fired and the degree of truth of

the consequence (θ_k) can be expressed as $\mu_i * \theta_j$.

Petri nets

Basic Petri nets

The concept of Petri net was firstly raised by Carl Adam Petri in 1962. Petri nets can be expressed both graphically and mathematically. Petri nets contain three basic elements, namely places, transitions, arcs, and one common-used element, tokens. Usually, in graphic, a circle represents a place, a rectangle or a bar represents a transition, a dot represents a token, a directed arrow represents an arc (Fig. 1). Arcs are only between a place and a transition-from a place to a transition or from a transition to a place. Tokens are hold within places and under some certain rules, the transition can be fired thus making some tokens moving from original places to other places. A token distribution represents a state of the system that Petri net models.

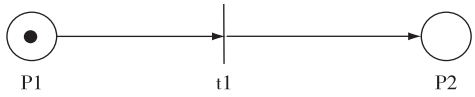

Fig. 1 Basic Petri net

The mathematical definition of Petri nets can been seen in many books and articles, such as defining Petri nets as 3-tuple (P, T, A), where P, T, A are the set of places, transitions and arcs respectively, and based on the basic Petri nets, a variety of Petri nets have been defined to meet the demand of modeling different systems to solve different problems, such as colored Petri net, time Petri net, fuzzy Petri net and so on.

Knowledge representation using Petri nets

As mentioned above, there are different Petri nets proposed, and also different Petri nets are used for knowledge representation including fuzzy Petri nets (FPN)[7,9 – 11], high-level fuzzy Petri nets[12], Pr/T net-systems [13], behavioral Petri nets [14] and so on.

Here, we introduce the fuzzy Petri nets knowledge representation in [7], which have been cited by many researchers and extended to different Petri nets. The fuzzy Petri net was defined as an 8-tuple:

$$FPN = (P, T, D, I, O, f, \alpha, \beta),$$

Where

$P = \{p_1, p_2, \cdots, p_n\}$ is a finite set of places,

$T = \{t_1, t_2, \cdots, t_m\}$ is a finite set of transitions,

$D = \{d_1, d_2, \cdots, d_n\}$ is a finite set of propositions, $|P| = |D|, P \cap T = \phi$,

$I: P \rightarrow T$ is the input function, a mapping from places to transitions,

$O: T \rightarrow P$ is the output function, a mapping from transitions to places,

$f: T \rightarrow [0,1]$ is an association function, a mapping from transitions to real values between zero to one,

$\alpha: P \rightarrow [0,1]$ is an association function, a mapping from places to real values between zero to one,

$\beta: P \rightarrow D$ is an association function, a mapping from places to propositions.

If P and D are considered to be the same by default, D and β can be deleted, and a simple 6-tuple can be got. And if more information needs to be captured, the 8-tuple can be extended like the 13-tuple and 9-tuple in [15].

It can be explained that P (places) represents D (propositions), which is propositions including antecedents and consequences, and α is a set of degrees of truth in accordance with propositions. T (transitions) represents a set of fuzzy rules and f is the set of Certainty Factors of rules. I (input function) and O (output function) are arcs between places and transitions which build fuzzy relationships between two propositions. Under this circumstance, only one token can be held within one place; the token's marking is a real value between zero to one which is the degree of truth corresponding to the place holding the token; and tokens cannot move from one place to another but their marking can be changed.

Therefore, the fuzzy production rule R_i: IF d_j THEN d_k ($CF = \mu_i$) can be modeled as shown in Fig. 2.

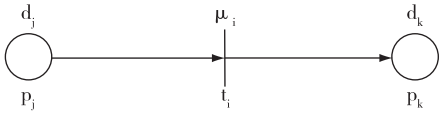

Fig. 2 A fuzzy Petri net

Reasoning algorithm

The reasoning process is to determine the degree of truth of consequences from the known degree of truth of antecedents, based on acquired knowledge. For reasoning a fuzzy Petri net, there exist many methods, and the two common-used methods are a reachability analysis method and a matrix equation analysis, which derive from two major Petri net analysis methods, the coverability tree and state equation, respectively.

The reachability analysis method was used in [7, 9]. This method builds a reachability set table and an adjacent place table according to the fuzzy Petri net model in the first place and the tree sprouts are generated towards the required answers. In [16], a fuzzy backward reasoning algorithm was developed to perform fuzzy backward reasoning to evaluate the degree of truth of any proposition specified by the user.

The matrix equation analysis method was adopted in [17 – 19]. The fuzzy Petri net can be expressed as

vectors or matrixes, so the algorithm based on matrix equations is quite simple and efficient to get the answers, and the only problem is that when the knowledge base is huge, the matrix may be enormous thus making the computation very slow.

Advantages of Petri nets knowledge representation

The advantages of Petri nets knowledge representation can be summarized as follows:

1) Petri nets provide a structural tool for model rule knowledge bases, and its graphical view makes it simple to see the relationships among rules and help to construct and modify rule bases.

2) Different kinds of Petri nets can be suitable for different application requirements. It is very feasible that users can design their own Petri nets based on achievements of former researchers.

3) After established, Petri nets can be analyzed efficiently so that if used in knowledge representation, the knowledge base can be verified through analyzing its Petri nets model so as to prevent conflicting, redundant, subsumed, circular, dead-end rules, which is of great significance to a knowledge base.

4) It is easy to develop efficient reasoning algorithm due to its concurrent feature, especially when matrix equations are used. In the Petri nets model, several rules can be applied in any order and fired in parallel, which makes the performance of an expert system better than traditional ones.

An illustration for diagnosing animal disease

When it comes to represent the animal disease knowledge, it is likely to use a composite fuzzy production rule containing AND/OR in most rules because most diseases have several main symptoms.

Assume there are three rules as follows:

R1: IF S1 AND S2 AND S3 THEN D1, CF = 0.95

R2: IF S1 AND S4 AND S5 THEN D2, CF = 0.90

R3: IF S3 AND S4 AND S6 THEN D3, CF = 0.80

S1, S2, S3, S4, S5, S6 means 6 symptoms and D1, D2, D3 means 3 diseases.

Its fuzzy Petri nets model is shown in Fig. 3.

Suppose that the degree of truth of symptoms (S1 to S6) got from detecting is 0.7, 0.2, 0.3, 0.7, 0.8, 0.3.

$FPN = (P, T, D, I, O, f, \alpha, \beta)$,

$P = \{P_1, P_2, P_3, P_4, P_5, P_6, P_7, P_8, P_9\}$,

$T = \{t_1, t_2, t_3\}$,

$D = \{S1, S2, S3, D1, S4, S5, D2, S6, D3\}$,

$I(t_1) = \{P_1, P_2, P_3\}$, $O(t_1) = \{P_7\}$, $I(t_2) = \{P_1, P_5, P_6\}$, $O(t_2) = \{P_7\}$,

$I(t_3) = \{p_3, p_5, p_8\}$, $O(t_3) = \{p_9\}$,

$f(t_1) = 0.95$, $f(t_2) = 0.90$, $f(t_3) = 0.80$,

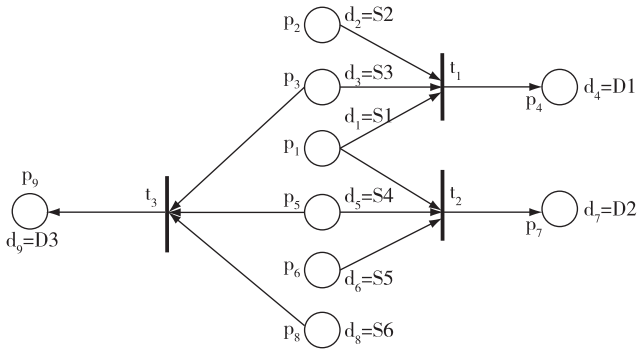

Fig. 3 Petri net representing rules

$\alpha(p_1) = 0.7$, $\alpha(p_2) = 0.2$, $\alpha(p_3) = 0.3$, $\alpha(p_4) = 0$, $\alpha(p_5) = 0.7$, $\alpha(p_6) = 0.8$,

$\alpha(p_7) = 0$, $\alpha(p_8) = 0.3$, $\alpha(p_9) = 0$,

$\beta(p_1) = S1$, $\beta(p_2) = S2$, $\beta(p_3) = S3$, $\beta(p_4) = D1$, $\beta(p_5) = S4$, $\beta(p_6) = S5$,

$\beta(p_7) = D2$, $\beta(p_8) = S6$, $\beta(p_9) = D3$.

Suppose that the threshold for antecedent is 0.3, the threshold for consequence is 0.6, which means if the degree of truth of an antecedent is greater than 0.3, the rule can be fired, and if the degree of truth of a consequence is greater than 0.6, the consequence can be regarded as true.

For a antecedent composed of several propositions in conjunction with AND, the degree of truth θ is determined by the minimum degree of truth of propositions. Therefore, in the above illustration, R1 cannot be fired since the degree of truth of D2 is 0.2, which is less than 0.3. And R2 and R3 can be fired. According to the firing of transitions in [7], the calculated degree of truth $\theta(D2) = 0.7 \times 0.9 = 0.63$, which is greater than the threshold 0.6 and D2 can be seen as true. The degree of truth $\theta(D3) = 0.3 \times 0.8 = 0.24$, which is less than the threshold 0.6 and D3 cannot be regarded as true from this point of view.

Of course, this is a very simple Petri nets modeling and if more information need to be included to make the system perform better, other modified or advanced Petri nets can be used as discussed above.

Conclusions

As discussed above, Petri net is a promising tool for knowledge representation due to its graphical, concurrent, dynamic and analytical features, and with regard to animal disease diagnosis expert systems, this undiscovered tool deserves researchers' attention who are dedicated to build a qualified animal disease diagnosis expert system. Based on the achievements that knowledge representation researchers have obtained, special Petri nets that suit the animal disease knowledge representation

can be developed so as to make a more efficient animal disease expert system.

Acknowledgement

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Sensitivity Profile Monitoring of Pathogens Isolated from Bovine Mastitis Subclinical Cases

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Summary: The efficacy *in vitro* of different antimicrobials against the mainly microorganisms enrolled in cases of subclinical bovine mastitis, isolated at Mastitis Research Nucleus, in the past five years, were studied. Results obtained indicated that cephalosporin and gentamicin were the more effective antimicrobials against more than 70% strains of the eight bacterial species/varieties evaluated. As both antimicrobials are commonly used for mastitis treatment with good results, we suggest that antibiogram can be kept as an additional tool for mastitis monitoring, treatment and control.

Introduction

Although bovine mastitis control be usually based on hygienic and sanitary measures, it is essential to adopt alternative actions for intra-mammary infection block. Antimicrobial therapy has as important role on its control, especially in mastitis clinical cases, in order to eliminate the infections causes by mainly microorganisms, avoiding its dissemination between animals. However, regional and technological modifications caused by human, associated to the selection of resistant microorganism strains inducted by intensively use of antimicrobials, have determined that mastitis become a higher problem, especially due the bacterial resistance phenomenon.

The treatment fails difficult the infection control on dairy herds. The monitoring of microbial sensitivity profile on farm can guide the treatment of subclinical mastitis cases at dry, and also for the clinical cases, immediately after its starting. Sensitivity profile study indicates the best antimicrobials that can be chosen for mastitis treatment in dairies; consequently contributing for the prognostic.

By other hand, it is important to point out that in some situations, although *in vitro* antimicrobial sensitivity studies be favorable, the treatment success may not be obtained, and several issues must be considered, as follows: the enrolled pathogen; the infection's severity and chronicity; the antimicrobial concentration and duration of use; the distribution of drug in the mammary gland; the medicine penetration on inflamed or injured areas of udder; the antimicrobial inactivation by milk and/or serum components, as the catabolic products resulted by infectious process; the intracellular location of some species of microorganisms, and also individual factors.

The aim of the present study was to evaluate the *in vitro* efficacy of different antimicrobials against the main microorganism enrolled in bovine mastitis subclinical cases, isolated at Mastitis Research Nucleus – NUPEMAS from Faculty of Veterinary Medicine and Animal Science – UNESP Botucatu-SP Brazil, in the past five years (from February 2008 to October 2012).

Material and methods

14,509 milk samples were examined, with 10,514 bacterial strains isolated from Holstein cows of variable ages and milking stages, in different regions of Sao Paulo State, Brazil. Eight bacterial species/varieties were evaluated, as follows: *Staphylococcus aureus*; coagulase negative *Staphylococcus* spp.; *Streptococcus agalactiae*; *Streptococcus dysgalactiae*; *Streptococcus uberis*; *Corynebacterium bovis*; *Escherichia coli* and *Klebsiella pneumoniae*.

After culture in ovine blood agar 8% and MacConkey agar, isolates were cultivated in brain-heart-infusion (BHI) for biochemical tests [1]. For antibiogram, they were cultivated in M ller-Hinton agar plates [2]. For *Corynebacterium bovis* growth, a 1% of Tween-80 was added to BHI. The antibiograms for this specific pathogen, and also for *Streptococcus* spp. were done in ovine blood agar 8% plates.

It were used disks of tetracycline (30 μg); neomycin (30 μg); penicillin G (10 UI); ampicillin (10 μg); gentamicin (10 μg); cephalosporin (30 μg); sulphazotrin (25 μg); erythromycin (15 μg); lyncomycin (2 μg); kanamycin (30 μg); streptomycin (10 μg), and enrofloxacyn (5 μg). It is important to reinforce that while *in vitro* susceptibility was documented in this study, there are regional differences in approved drugs for treatment of dairy cattle.

Results and discussion

Detailed results about sensitivity profile of the main microorganisms isolated against the different evaluated antimicrobials are shown on Table 1 and 2.

Table 1 Efficacy *in vitro* of different antimicrobials against the main microorganisms cultured from cases of subclinical bovine mastitis, isolated at Mastitis Research Nucleus – NUPEMAS, from February 2008 to October 2012. Botucatu, Sao Paulo, Brazil, 2012.

Antimicrobial	*S. aureus* N = 2.520		CNS N = 1.850		*Str. agalactiae* N = 1.580		*Str. dysgalactiae* N = 1.128	
	S	%	S	%	S	%	S	%
Tetracycline	1.058	42.0	536	28.9	442	27.9	394	34.9
Neomycin	1.915	76.0	1.350	730	759	48.0	507	44.9
Penicillin G	352	14.0	333	18.0	1.311	82.9	893	79.2
Ampicillin	1.134	45.0	777	42.0	1.232	77.9	803	71.2
Gentamicin	2.242	89.0	1.498	81.0	774	48.9	518	45.9
Cephalosporin	2.217	88.0	1.554	84.0	1.327	83.9	953	84.5
Sulphazotrin	1.638	65.0	1.036	56.0	1.058	66.9	699	61.7
Erythromycin	1.713	67.9	1.073	580	1.279	81.0	561	49.7
Lyncomycin	1.335	52.9	999	54.0	711	45.0	462	41.0
Kanamycin	1.310	52.0	906	48.9	726	459	460	40.8
Streptomycin	478	18.9	388	20.9	331	20.9	253	22.4

N = number of samples; *S. aureus* = *Staphylococcus aureus*; CNS = coagulase negative *Staphylococcus* spp.; *Str. agalactiae* = *Streptococcus agalactiae*; *Str. dysgalactiae* = *Streptococcus dysgalactiae*; S = number of samples that presented sensitivity to the tested antimicrobial; % = percentage of samples presented sensitivity to the tested antimicrobial.

Table 2 Efficacy *in vitro* of different antimicrobials against the main microorganisms cultured from cases of subclinical bovine mastitis, isolated at Mastitis Research Nucleus – NUPEMAS, from February 2008 to October 2012. Botucatu, Sao Paulo, Brazil, 2012.

Antimicrobial	*Str. uberis* N = 858		*C. bovis* N = 1.981		*E. coli* N = 315		*K. pneumoniae* N = 282	
	S	%	S	%	S	%	S	%
Tetracycline	223	26.0	1.168	58.9	129	41.0	157	55.6
Neomycin	271	31.6	812	41.0	234	74.3	180	63.8
Penicillin G	673	78.4	574	29.0	NT	NT	NT	NT
Ampicillin	631	73.5	916	46.2	250	79.4	242	85.8
Gentamicin	417	48.6	1.624	82.0	239	75.9	222	78.7
Cephalosporin	665	77.5	1.287	64.9	223	70.1	224	79.4
Sulphazotrin	592	69.0	951	48.0	233	74.0	208	73.7
Erythromycin	653	76.1	1.612	81.4	217	68.9	201	71.3
Lyncomycin	387	45.1	832	42.0	212	67.3	161	57.0
Kanamycin	343	40.0	1.406	70.9	239	75.8	197	69.8
Streptomycin	163	19.0	NT	NT	245	77.8	183	64.8
Enrofloxacyn	NT	NT	NT	NT	286	91.0	250	88.6

N = number of samples; *Str. uberis* = *Streptococcus uberis*; *C. bovis* = *Corynebacterium bovis*; *E. coli* = *Escherichia coli*; *K. pneumoniae* = *Klebsiella pneumoniae*; S = number of samples that presented senstitivity to the tested antimicrobial; % = percentage of samples presented senstitivity to the tested antimicrobial; NT = non-tested.

The following antimicrobials were effective against more than 70% of the examined strains: neomycin, gentamicin and cephalosporin for *Stahylococcus aureus*; neomycin, gentamicin and cephalosporin for Coagulase Negative *Staphylococcus* spp.; penicillin G, ampicillin, cephalosporin and erythromycin for *Streptococcus agalactiae*; penicillin G, ampicillin and cephalosporin for *Streptococcus dysgalactiae*; penicillin G, ampicillin, cephalosporin and erythromycin for *Streptococcus uberis*; gentamicin, erythromycin and kanamycin for *Corynebacterium bovis*; neomycin, ampicillin, gentamicin, cephalosporin, sulphazotrin, kanamycin, streptomycin and enrofloxacyn for *E. coli*; ampicillin, gentamicin, cephalosporin, sulphazotrin; erythromycin and enrofloxacyn for *Klebsiella pneumoniae*. Detailed data about the more effective *in vitro* antimicrobials against the eight species/varieties of microorganisms evaluated are shown on Table 3.

It is important to point out that the two more effective *in vitro* antimicrobials against the bacterial species/

varieties evaluated were cephalosporin and gentamicin. These drugs are commonly used for mastitis treatment in dairy cows, due its large spectrum of action, with high efficacy against the main pathogens enrolled in clinical and subclinical cases. There are different available intra-mammary commercial presentations that contain these antimicrobials, and the treatment indication depending on its concentrations.

Table 3 More effective *in vitro* antimicrobials against the examined strains isolated from subclinical cases of bovine mastitis at Mastitis Research Nucleus – NUPEMAS, from February 2008 to October 2012. Botucatu, Sao Paulo, Brazil, 2012.

Antimicrobial	Total of sensible bacterial strains species*	%**
Cephalosporin	7	87.5
Gentamicyn	5	62.5
Ampicillin	5	62.5
Erythromicyn	5	62.5
Neomicyn	3	37.5
Penicillin G	3	37.5
Sulphazotrin	2	25.0
Kanamicyn	2	25.0
Enrofloxacyn	2	25.0

* Number of species which had more than 70% sensible strains to the antimicrobial. Total of evaluated species/varieties: 8 (*Staphylococcus aureus*; coagulase negative *Staphylococcus* spp.; *Streptococcus agalactiae*; *Streptococcus dysgalactiae*; *Streptococcus uberis*; *Corynebacterium bovis*; *Escherichia coli* and *Klebsiella pneumoniae*).

** Percentage of sensible species to the antimicrobial considering the total of eight evaluated species.

First generation cephalosporins are the most indicated, especially in subclinical cases and for treatment of cows at dry. These drugs have better action against Gram positive microorganisms, which are responsible for the main part of subclinical mastitis cases. Also, in acute clinical cases, they have shown excelent results against *Escherichia* coli and *Klebsiella* sp. [3].

Conclusions

Although *in vitro* antimicrobial sensitivity profile studies are not totally correlated with the *in vivo* efficacy of drugs, due external and internal factors involved, the results obtained in the present study reinforce the satisfactory efficacy of some large spectrum antimicrobials like cephalosporin and gentamicin against the main pathogens isolated from bovine subclinical mastitis cases. As these are commonly used for mastitis treatment with satisfactory results, we conclude that the antibiogram test can be kept as an additional tool for treatment guide in order to reach the mastitis control in dairy herds.

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Effect of Source and Supplementation Time of Dietary Fiber on Performance of Gestation Sows

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Summary: This paper investigated effects of replacing of wheat bran with a commercial fiber product started from different physiological periods of gestation on performance of sows. Total of 60 Landrace × Large Yorkshire primiparous sows were selected and randomly divided into five groups. The sows in the control group were fed with basic gestation diet with wheat bran as major source of crude fiber. Those in experimental groups T1 to T4 were fed with basic gestation diet followed by experiment diet with 2% Arbocell® instead of wheat bran started from the 91st day, 61st day, 31st day and 1st day after mating, respectively. The result showed that average daily feed intake of sows decreased linearly and quadratically during gestation period and increased linearly and quadratically during lactation period as we gradually shift to earlier time to substitute dietary fiber Arbocell® for wheat bran. At the same time, delivery periods decreased linearly, wean to estrus intervals decreased linearly and quadratically, born alive and born weight increased linearly, born alive percentage increased linearly, weaning alive and weaning weight tended to increase linearly, weaning rate tended to increase cubically, and average daily gain of piglets increased cubically, feces excretion time and feces weight of sows decreased linearly and cubically. The astriction scoring of sows decreased significantly. In conclusion, the alternative fiber product substituted for wheat bran is effective for sows' healthy production. The earlier the dietary fiber employed the better the sows' performance appeared.

Introduction

Sows are normally fed a restricted diet during pregnancy and supplied *ad libitum* after parturition to prevent excessive weight gain and to optimize their milk yield[1 – 3]. The spontaneous ingestion of young hyper prolific sows often appears insufficient to respond to the needs of lactation and the maintenance of body reserves, but also to ensure a satisfactory course for the next reproductive cycle. The effects of high fiber levels in diets for pregnant sows were studied in many experiments[4 – 6]. The availability of a high-fiber diet during gestation may reduce frustrations due to feed restrictions in gestating sows and may better prepare them for a more substantial post-partum diet. This experiment was designed to check the effects of replacing of wheat bran with a commercial fiber product started from different physiological periods of gestation on performance of sows.

Material and methods

Total of 60 Landrace Large Yorkshire primiparous sows, near mating date, were selected and randomly divided into five groups. The study and animal care protocol followed the guidelines from the Laboratory of Animal Management Association of Nanjing Agricultural University. The sows in the control group (C) were fed with basic gestation diet with wheat bran as the major source of crude fiber. Those in experimental groups were fed with basic gestation diet followed by experiment diet with 2% Arbocell® instead of wheat bran (Arbocel® is a fiber product provided by J. Rettenmaier & Söhne GmbH CO.) started from the 91st day, the 61st day, the 31st day and the 1st day after mating. All sows fed with basic lactation diet after weaning. Sows were fed twice daily. Piglets were taught to eat creep feed since 7 days of age, fed and watered *ad libitum*, weaned at 28 days of age. Feed consumption of the experimental pigs was collected as follows: Weight of feeds given to experimental pigs was recorded daily. Surplus feeds remained in the feeders were cleared away and weighed daily. Feed intake of experimental pigs was hence calculated. Parturition status (including duration of parturition, total litter size, weak, stillbirth, mummy, alive litter size, etc.), body composition, feces consistency, lactation, MMA, et al were observed and recorded. Body weight at birth, 7 d, 20 d, 28 d (weaning), feed intake of piglets were also observed and recorded. Feces samples were collected and astriction scoring was judged as follows: The sow frequently evacuated cylindrical-shaped and soft feces was identified as no astriction (score: 0). The one frequently evacuated large particle and soft feces was identified as slight astriction (score: 1). The one seldom evacuated with large particle and a bit soft feces was identified as moderate astriction (score: 2). The one evacuated difficultly even though she tried frequently with

granulated, large and hard feces was identified as less severe astriction (score: 3). The one evacuated difficultly even though she tried frequently with little, granulated, grape shaped and very hard feces was identified as severe astriction (score: 4). At the same time, urine sample was also collected and pH value was tested.

Data of all values were expressed in the form of least square means. All statistical analyses were performed as a randomized complete block design using GLM procedures of SAS and significant differences were examined by least significant different (LSD) test. Pen was considered as the experimental unit for all the data. Difference for probability level of $P \leqslant 0.05$ was considered as statistically significant while $P \leqslant 0.10$ was considered as a tendency.

Table 1 Effect of fiber product ARBOCEL® on performance of sows

Item	CT	T1	T2	T3	T4	P value		
	12	12	12	12	12	linear	quadratic	cubic
Feed intake of gestation sows, kg	2.30	2.30	2.21	2.19	2.11	<0.01	<0.01	0.24
Feed intake of lactation sows, kg	4.66	4.94	5.22	5.47	5.70	<0.01	<0.01	0.31
Feces excretion time of sows, sec	60.70	51.96	52.46	49.19	37.14	<0.01	<0.01	<0.01
Feces weight of sows, kg	0.26	0.35	0.27	0.28	0.23	0.57	<0.01	<0.01
Astriction scoring	2.16	1.30	1.21	1.50	0.54	<0.01	<0.01	0.33
Urination time of sows, sec	42.70	41.56	39.32	40.31	39.54	<0.01	0.06	0.09
Urine pH of sows	7.39	7.39	7.40	7.39	7.43	0.94	0.77	0.82
Delivery periods, min	266.17	212	181	154	141.2	0.02	0.06	0.61
Periods between piglets born, min	21.31	17.68	20.95	13.77	15.54	0.25	0.36	0.77
Total born number	11.17	11.00	11.08	11.17	11.67	0.97	0.81	0.91
Born alive	8.67	9.83	10.08	10.42	11.33	0.03	0.46	0.69
Weaning alive	8.33	8.67	9.58	9.75	10.50	0.07	0.67	0.44
Weaning rate	96.92	88.73	96.96	92.42	93.59	0.73	0.60	0.06

Result and disscusion

As supplementation beginning time of dietary fiber Arbocell® to substitute wheat bran shifted from none, 91 d, 61 d, 31 d to 1 d after insemination, average daily feed intake of sows decreased linearly and quadratically ($P < 0.01$) during gestation period and increased linearly and quadratically ($P < 0.01$) during lactation period (Table 1). Delivery periods decreased linearly ($P < 0.01$). Wean to estrus intervals decreased linearly ($P < 0.01$). Born alive increased linearly ($P < 0.05$). Born alive percentage increased linearly ($P < 0.01$). Born weight increased quadratically ($P < 0.05$). Weaning alive and weaning weight tended to increase linearly ($P < 0.10$). Weaning rate tended to increase cubically ($P < 0.10$). Average daily gain of piglets increased cubically ($P < 0.05$). This of course confirms that the earlier the usage of the fiber the better the sows' performance. Researches of dietary fiber on gestating sows were majorly focused on effects on parturition progress, behavior, reproductive performance, and nutrient digestibility in gestating sows[7 – 9]. Ramonet et al. [5] showed that a high-fiber diet (12.9 MJ DE/kg of DM, 18.1% CF) can reduce apparent feeding motivation of pregnant sows and, thus, improve the welfare of sows subjected to feed restriction. Holt et al. [7] reported that a high-fiber diet (2.80 kg/day, 12.4% crude fiber in% of dry matter) during gestation did not influence duration of gestation and weaning to estrus interval, as well as piglet weight at birth, litter size, number of stillborn and weaned piglets, but improved 1st week growth rate and tended to increase weaning weight of piglets. Quesnel et al. [10] investigated that feeding sows a bulky diet contained 11.0% crude fiber during gestation did not differ gestation sows' body weight or backfat gain while increased lactation sows' average diet intake and increased piglets growth rate. Almost all the studies applied the experimental diet throughout the whole periods of gestation.

Very few researches were made to study effect of dietary fiber on healthy situation of sows till now. In our present research, feces samples were collected and astriction scoring was judged, urine sample was also collected and pH value was tested. As we gradually shift to earlier time to substitute dietary fiber Arbocell® for wheat bran, feces excretion time of sows decreased linearly and cubically ($P < 0.05$) during gestation period and lactation period. Feces weight of sows decreased quadratically and cubically ($P < 0.01$) during gestation period and lactation period. The feces judgment grade of sows decreased significantly. The astriction scoring judgment grade of sows showed linearly decreased ($P < 0.01$). The urinating time of sows decreased linearly either ($P < 0.05$).

Conclusions

In conclusion, the alternative fiber product substituted for wheat bran is effective for sows' healthy production considering the parturition performance and fertility of the sows, feces and urination excretion, and growth performance of their piglets. The earlier the dietary fiber employed the better the sows' performance appeared.

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Influenza Virus A/Beijing/501/2009(H1N1) NS1 Interacts with β-Tubulin and Induces Disruption of the Microtubule Network and Apoptosis on A549 Cells

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Summary: NS1 of influenza A virus is a key multifunctional protein that plays various roles in regulating viral replication mechanisms, host innate/adaptive immune responses, and cellular signalling pathways. These functions rely on its ability to participate in a multitude of protein-protein and protein-RNA interactions. To gain further insight into the role of NS1, a tandem affinity purification (TAP) method was utilized to find unknown interaction partner of NS1. The protein complexes of NS1 and its interacting partner were purified from A549 cell using TAP-tagged NS1 as bait, and co-purified cellular factors were identified by mass spectrometry (MS). We identified cellular β-tubulin as a novel interaction partner of NS1. The RNA-binding domain of NS1 interacts with β-tubulin through its RNA-binding domain, as judged by a glutathione S-transferase (GST) pull-down assay with the GST-fused functional domains of NS1. Immunofluorescence analysis further revealed that NS1 with nucleolin co-locatized in the nucleus. In addition, the disruption of the microtubule network and apoptosis were also observed on NS1-transfected A549 cells. Our findings suggest that the 2009 pandemic H1N1 virus may utilize its NS1 protein to interact with cellular β-tubulin, further disrupt normal cell division and induce apoptosis, thereby facilitate virus replication and indirectly contribute to virus pathogenicity.

Introduction

Influenza A viruses are globally important human and animal respiratory pathogens that are responsible for both seasonal, endemic outbreaks, and periodic unpredictable world-wide pandemics [1]. Three human pandemics occurred during the last century [2]. The worst influenza A pandemic on record in 1918 killed approximately 50 million people worldwide [3]. In 2009, a novel swine-origin H1N1 influenza virus emerged in Mexico and quickly spread to other countries, including China. According to the WHO statistics, the virus has killed more than 18000 people. Influenza A virus belongs to the orthomyxoviridae family along with influenza viruses B and C. The influenza A virion is an enveloped RNA virus of spherical to ovoid shape measuring 80 – 120 nm in diameter. They contain a single-stranded, negative sense, segmented RNA genome consisting of eight segments of viral RNA (vRNA), which encode 11 known proteins [4]. The non-structural protein 1 (NS1) is the most important viral regulatory factor during infection. It is translated from a transcript of the segment eight and. plays various roles in regulating viral replication mechanisms, host innate/adaptive immune responses, and cellular signalling pathways. All of these functions of NS1 rely on its ability to participate in a multitude of protein-protein and protein-RNA interactions [5]. To date, over twenty cellular factors have been described which interact with NS1. These include RIG-I [6], poly(A)-binding protein I (PABPI) [7], p85b [8], importin-a, nucleolin [9], NS1-BP [10], eIF4GI [7], hStaufen[11], NS1-I[12], PKR [13], PACT, CPSF30[14], poly(A)-binding protein II (PABPII) [15], Crk/CrkL [16], PDZ domain-containing proteins [5], the viral polymerase, and components of the cellular mRNA nuclear export machinery (E1B-AP5, p15, NXF1, and Rae1) [17] However, in view of the extreme multifunctional nature of NS1, more cellular factors maybe need to associate to this protein so as to fulfill different functions. It is reasonable to assume there are other unidentified interaction partners of NS1 protein.

Despite our substantial knowledge of this amazing and fascinating protein, much still remains to be learnt of its roles in the virus replication cycle. To gain further insight into the role of NS1, we tried to find novel cellular factors

that interact with NS1. By utilizing a tandem affinity purification (TAP) system, we identified a cellular factor, β-tubulin, as new interaction partner of NS1 protein. In addition, the disruption of the microtubule network and apoptosis were also observed on NS1-transfected A549 cells. Our finding suggested that NS1 affects cellular functions through interaction with β-Tubulin.

Material and methods

A549 (ATCC CCL-185) cells were grown in DMEM supplemented with 10% FBS, 100 IU penicillin and 100 μg/ml streptomycin. The total RNA of influenza strains A/Beijing/501/2009 (H1N1) was kindly provided by Dr. Bohua Liu. The NS1 cDNA was amplified by RT-PCR and were ligated between the *Bam*H I and *Eco*R I sites of pnTAP vector; The correct sequence of all constructs described above was verified by sequencing. NS1-TAP protein complexes were expression and purification. To characterize the TAP-purified protein, the protein bands were excised from the Coomassie Blue-stained SDS-PAGE gel, in-gel digested by trypsin, and analyzed by MALDI-TOF mass spectrometer AXIMA-QIT. Proteins were identified from peptide fragments by comparison to theoretical digests of the human proteome using MASCOT search tools. To exclude the possibility that the interacting partner might represent unspecific factor, and further confirm the specific interaction, co-immunoprecipitation experiments were performed. To determine whether apoptosis was induced in the A549 cells transfected with influenza virus A/Beijing/501/2009 (H1N1) NS1, coverslips with adherent transfected A549 cells were collected and washed, and the A549 cells were fixed and stained with Hoechst 33342. The coverslips were washed, mounted on glass slides, and stored at 4℃ until quantification by fluorescence microscopy could be performed. To determine which domain of NS1 binds with β-tubulin, GST-fused NS1 constructs were prepared. NS1 fragments were amplified and was digested with *Bam*H I and *Sma* I and cloned into *Bam*H I-*Sma* I digested pGEX-4T1 vector. A549 cells were lysed and then centrifuged. The supernatant was collected and used as the total cell lysate. Each GST-fused NS1 was added into the total cell lysate, then the mixture was incubated with glutathione Sepharose beads. After extensive washing, the bound materials were eluted. The eluate was resolved on SDS-PAGE and analyzed by immunoblotting using anti-β-tubulin.

Results and discussion

The purified protein complexes were separated on 1-D polyacrylamide gels and stained with Coomassie Blue, then the protein bands were excised. The representative result is shown in Fig. 1A. As confirmed by western blot, NS1 and interacting partners were major in the soluble portion of whole cell extract (Fig. 1B). When the pattern of protein bands was compared between TAP-NS1 complexes and TAP-Null complexes, one major band (about 55 kDa, indicated by asterisk) was specific to the TAP-NS1 complexes on Coomassie Blue-stained gel. Although there are some other minor bands, we focus on the 55 kDa band. To identify this protein, the observed 55 kDa band was excised from the gel, subjected to trypsin digestion, and analyzed using a MALDI-TOF mass spectrometer. The peptide mass fingerprinting pattern of the band was obtained successfully and applied to query a database search engine, MASCOT. The best match of the 55 kDa protein was with β-tubulin, a multifunctional cytoskeletal protein (Fig. 2A). Overall, 12 peptides, ranging in size from 8 to 19 amino acids, were matched, representing a 33% sequence coverage of β-tubulin (141 of a total of 426 amino acids). By immunoblotting using an anti-β-tubulin monoclonal antibody, we confirmed that the detected 55 kDa band was β-tubulin in the TAP-NS1 complexes (Fig. 2B). β-tubulin was not detected in parallel analysis with the TAP-Null complexes (mock) (Fig. 2B). These results suggested that β-tubulin and NS1 are associated in transfected A549 cells and pulled down together. The association of β-tubulin and NS1 in A549 cell lysate was further confirmed by conventional co-immunoprecipitation using an anti-HA rabbit polyclonal antibody. As expected, TAP-NS1 was detected in the precipitates of A549 cells co-tranfected with pnTAP-NS1 and pCMV5-HA-β-tubulin by the anti-CBP

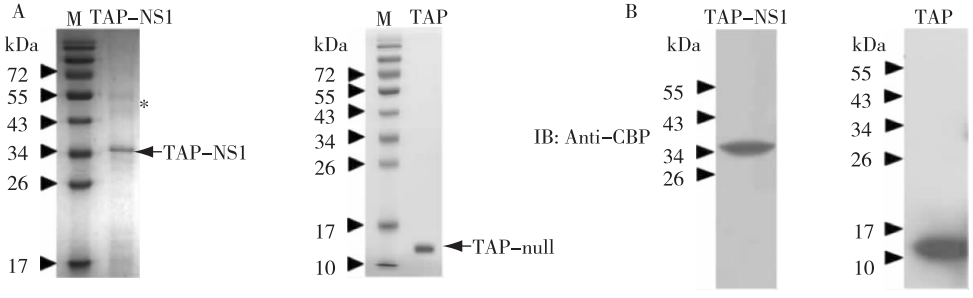

Fig. 1 Purification of cellular interaction partner of influenza strains A/Beijing/501/2009(H1N1) NS1

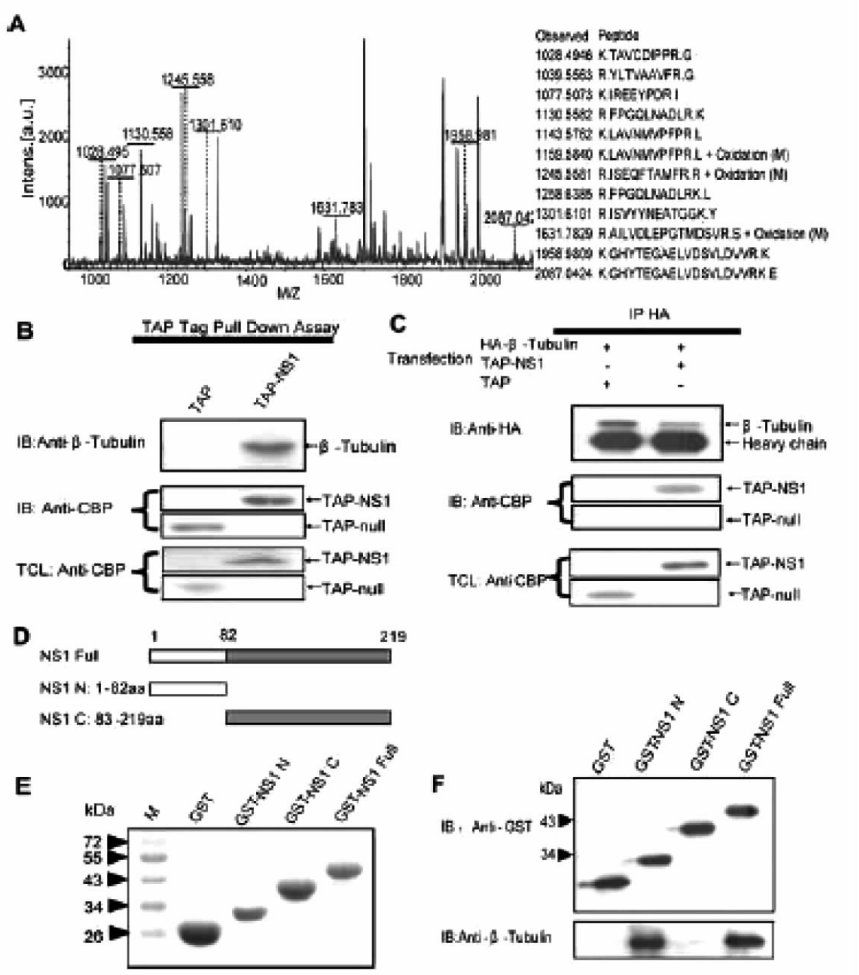

Fig. 2 Identification of β-tubulin as a novel NS1-binding protein. (A) Peptide mass fingerprinting of the 55 kDa protein.

antibody (Fig. 2C), whereas in control co-immunoprecipitation using pnTAP vector and pCMV5-HA-β-tubulin co-transfected cells, no TAP-NULL was detectable. In summary these results indicate that β-tubulin specifically associate with the NS1.

To determine which functional domain of NS1 directly interacts with β-tubulin, a GST pull-down assay was performed. Each fragment of influenza virus A/Beijing/501/2009 (H1N1) NS1, full-length (amino acids 1-219, GST-NS1 full), the N terminal domain (amino acids 1-81, GST-NS1 N), and the C terminal domain (amino acids 82-219, GST-NS1 C), was fused with GST and incubated with untransfected A549 cells lysate, and complexes were then pulled down using glutathione Sepharose. As seen in the results (Fig. 2D – F), β-tubulin was pulled down with GST-NS1 full or GST-NS1 N but not with GST-NS1 C, indicating that the N terminal domain of NS1 is responsible for binding with β-tubulin (Fig. 2F).

To clarify the distribution of NS1 and β-tubulin and their relationship in transfected cells, A549 cells were transfected with pCMV5-HA-NS1 and control vector pCMV5 respectively, and then fixed with 4% paraformaldehyde. To visualize NS1 and β-tubulin, a double-immunofluorescence staining was carried out. NS1 was apparent from 24 h post-transfection, mainly in nucleus (green color) (Fig. 3E). On the other hand, β-tubulin was stained in nucleus and cytoplasm (red color) (Fig. 3F). The signals of NS1 and β-tubulin clearly overlapped in nucleus, indicating that NS1 and β-tubulin co-localize in the nucleus of A549 cell (Fig. 3G).

To detect whether apoptosis was induced in the A549 cells transfected with influenza virus A/Beijing/501/2009 (H1N1) NS1, the cells were then washed, fixed with 4% paraformaldehyde, and stained with Hoechst 33342 for 20 min at 25℃. The slides were then examined by fluorescence microscopy and photographed. As illustrated in Fig. 3D and 3H, A549 cells transfected with influenza virus A/Beijing/501/2009 (H1N1) NS1 for 24 h and stained with Hoechst 33342 exhibited a stronger blue fluorescence and condensated and fragmented nuclear after expression NS1 for 24 hours.

In addition, an intriguing phenomenon was observed in immunofluorescence staining test. Transfection of A549

cells with plasmid pCMV5-HA-NS1 induced visible changes in microtubule structure in accordance with the disassembly of tubulin polymers at 24 h post transfection (Fig. 3F), whereas mock transfection of A549 cells with pCMV5 vector did not induce perceptible changes in microtubule structure. All these indicated that influenza virus A/Beijing/501/2009(H1N1) NS1 induce apoptosis and depolymerization of the microtubule network in A549 cell.

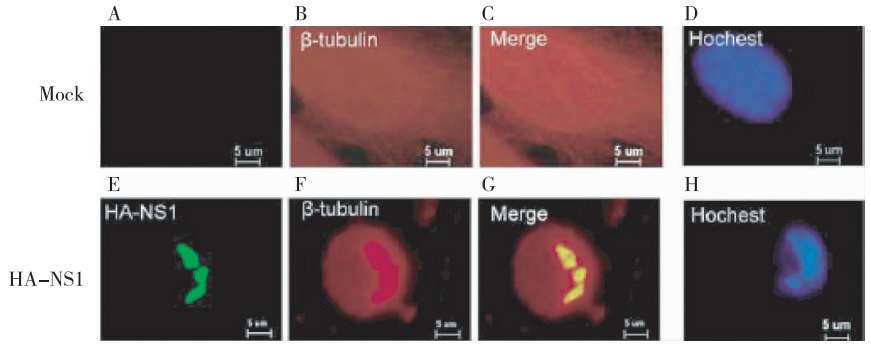

Fig. 3 Co-localization of NS1 and β-tubulin in the nucleus, and Influenza virus A/Beijing/501/2009(H1N1) NS1 induce apoptosis

Conclusion

In summary, the present study provides evidence that β-tubulin represent a novel interaction partner of influenza A virus NS1 protein. The RNA-binding domain of NS1 is responsible for binding with β-tubulin. The interaction of NS1 with β-tubulin disrupts the cellular microtubule network and induces apoptosis on human A549 cells. Our findings suggest that the 2009 pandemic H1N1 virus may utilize its NS1 protein to interact with cellular β-tubulin, further disrupt normal cell division and induce apoptosis, thereby facilitate virus replication and indirectly contribute to virus pathogenicity.

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Whole Genome Sequencing and Biological Characterization of Duck/JS/10, a New Lentogenic Class Ⅰ Newcastle Disease Virus

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Abstract: A lentogenic Newcastle disease virus (NDV), Duck/JS/10 (JS10), was isolated from a non-vaccinated duck in China. The complete genome of the virus contained 15,198 nucleotides. Based on length of genome and sequence of partial F gene, the virus was classified as a class Ⅰ genotype 4 NDV. The antigenicity of the virus was compared with that of NDV strain La Sota via hemagglutination inhibition (HI), virus neutralization (VN) assay and animal experiment. Our results show that JS10 generates higher HI and VN titers than La Sota against both class Ⅰ and Ⅱ virulent NDV strains. Experiments on animals demonstrate that virus shedding from chickens vaccinated with JS10 is significantly reduced when compared to those vaccinated with La Sota. Overall, the study strongly suggests that JS10 may qualify as a new vaccine candidate against Newcastle disease.

Key words: class Ⅰ Newcastle disease virus, genome sequence, immunogenicity, vaccine candidate

Inhibition of Foot-and-Mouth Disease Virus Replication by the Bovine Mx1 Protein

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Abstract: Foot-and-mouth disease virus (FMDV) is the most economically important veterinary pathogen that causes Foot-and-mouth disease (FMD), which is a sometimes fatal viral disease because of its highly infectious nature, ability to cause persistent infections and long term effects on the condition and productivity of animals with cloven hooves. Among the antiviral proteins that are synthesized in response to interferon (IFN) stimulation *in vivo*, Mx proteins from several species which belong to the dynamin superfamily of large GTPase are long known to block the replication of a wide range of RNA viruses, such as the high pathogenic avian influenza virus (AIV), Thogoto virus (THVO) and vesicular stomatitis virus (VSV). It was reported that bovine Mx1 protein is an IFN-induced cytoplasmic protein with powerful antiviral activity against vesicular stomatitis virus (VSV) and rabies virus. This study try to exeamine whether bovine Mx1 protein could inhibit replication of FMDV. This question was addressed by inoculating FMDV serotype O onto a bovine Mx1 expressing BHK-21 cell clone. After 48 h, the copies of FMDV VP1 gene waere detected by real-time reverse transcriptase PCR, it was profoundly reduced in boMx1-expressing clones by 73.5%. Then supernatants were sampled and their titers was determined onto BHK cell monolayers by the 50% tissue culture infective dose method (TCID50), viral yields were reduced 10- to 100-fold by bovine Mx1 expression. These results demonstrate that bovine Mx1 protein has a powerful antiviral activity against FMDV. Alternatively, bovine Mx1 could be unique in its ability to FMDV which, if confirmed *in vivo*, would provide the basis for the Mx transgenic animal preparation and the disease-resistant breeding.

Pathological and Etiological Study of the Swine High Fever Syndrome (SHFS) in Central Region of Shandong Province

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Abstract: To study the pathological changes and the etiological agent of the Swine High Fever syndrome (SHFS) in central region of Shandong Province, we diagnosed the Clinical symptoms and the pathological changes of 109 cases of 56 pig farms and collected 363 serum samples from Jan. 2011 to Mar. 2012. . (Method) Three viruses were detected on tissue samples by histopathological, immunohistochemical examination and PCR or RT-PCR, including classical swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus(PRRSV), porcine circovirus type 2 (PCV-2). Serological tests were used for detecting prevalence of CSFV, PRRS, and PCV-2 antibody by ELISA. (Result) The results showed that all pigs suffering from high fever had been vaccinated by Classical Swine Fever vaccine, part of them by porcine reproductive and respiratory syndrome attenuated live vaccines, but none of them vaccinated by Circovirus. While they all have higher antibody positive rate. The detection rates of the lymphoid tissue with acute inflammation, interstitial pneumonia, viral encephalitis were highest, respectively 92.3% ,76.1% and 66.1% . sometimes there were mixed bacterial infections . The pathogenetic rates of CSFV, PRRSV and PCV2 were respectively 30.27% , 66.97% , and 41.28% . The co-infection rates of CSFV and PRRSV, CSFV and PCV2, PRRSV and PCV2 were respectively 16.51% ,6.42% and 28.44% . And the triple infection rate was 4.59% in addition other pathogenic infections were 8.26% . Sequence analysis showed that the epidemic strains of CSFV were developing towards being far away from the HCLV, and all PRRSV strains were highly pathogenic ones. Also PCV2 was mainly the virulent Type-PCV2b . According to the results, CSFV, PRRSV and PCV2 were the main pathogens which caused the Swine High Fever in central region of Shandong province, and co-infection was the main reason to the severe cases. Sometimes Mycoplasma and Haemophilus parasuis mixed infection in clinical cases. Therefor multiple infection led to the Swine High Fever in central region of Shandong Province.

Establishment and Application of TaqMan-MGB Fluorescence Quantitative PCR for Detection of Pseudorabies Virus

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Abstract: A pairs of primers and a TaqMan-MGB probes were designed and synthesized according to the the nucleotide sequence of the gE gene of pseudorabies virus (PRV) available in GenBank, and real-time TaqMan-MGB fluorescence quantitative PCR for distinguishing the wild strain and gene-deleted vaccine strain of PRV was established successfully. It was demonstrated that the established TaqMan-MGB quantitative PCR assay could detect 2.23×10^1 copys·μL^{-1} of plasmid DNA. Sensitivity and positive rate for clinical sample of TaqMan fluorescent quantitative PCR were higher than routine PCR, and its sensitivity was 100 times higher than that of the routine PCR., and had no cross reaction with classical swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine cireovirus type 2 (PCV2) and porcine parvovirus (PPV). The 30 suspected material were detected by TaqMan-MGB fluorescence quantitative PCR and routine PCR, respectively, the positive detection rate were 40% and 33%, the coincidence rate was 90%. The real-time TaqMan fluorescence quantitative PCR assay which is specific, sensitive, rapid and accurate can be used for the diagnosis of PRV infection.

Designing of Injectable Dosage Form for the Treatment and Prevention of Liver Disease in Animals

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Abstract: This paper discusses the design of injectable dosage form for the treatment and prevention of liver disease in animals and study the possibility of using it for complex treatment of liver pathologies.

It is known that the liver is the central organ of metabolism, which takes most of the chemical processes involved in the metabolism of proteins, carbohydrates, lipids, vitamins and minerals. The liver is actively involved in digestion, detoxification of toxic substances from the gastrointestinal tract, and enter the body from the outside, homeostasis, etc. Therefore compromising its functional activity leads to a rather substantial disruption of vital activity. All this makes it relevant to the search for effective drugs or combinations that reduce risk and implement effective therapy liver pathologies associated with environmental factors. It is important that medicines are non-toxic and exhibit increased bioavailability.

The goal of our research is to develop a stable injectable drug containing silymarin, which will increase the bioavailability and reduce side effects. This problem is solved by the invention, injection dosage form for the treatment and prevention of liver disease in animals contains silymarin, an organic solvent, solubilizer, preservative and co-solvent in the following ratio, wt. %: silymarin 1 – 10, organic solvent 5 – 50, solubilizer 3 – 30, preservative 0. 01 – 1, cosolvent else.

The technical result of our research is to provide a stable injectable silymarin with sufficiently low viscosity, thus reducing painful injections. The invention relates to the field of pharmaceuticals and can be used for the treatment of liver abnormalities in animals. The dosage form contains silymarin, an organic solvent, solubilizer, preservative and co-solvent in the following ratio, wt. %: Silymarin 1 – 10, an organic solvent 5 – 50, solubilizer 3 – 30, preservative 0. 01 – 1, co-solvent - the rest. Injectable form stable, non-toxic, has no local irritant and allergenic properties, will increase the bioavailability of silymarin and reduce side effects.

Chronic Toxicity Study of Injection Drug for the Treatment and Prevention of Liver Disease in Animals

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Abstract: This paper deals with the study of chronic injectable drug for the treatment and prevention of liver disease in animals and study the possibility of using it for complex treatment of liver pathologies.

Chronic toxicity was studied in 18 white male mice with an initial mass of 20 – 25, all the animals were divided into 3 groups of 6 animals each. The animals of the experimental group 1 intramuscularly daily for 1 month study medication was administered at a dose of 10 mg/kg for the active substance or 0. 8 ml/kg (concentration of active ingredient in the drug was 12 mg/ml), which corresponded to 50 1/10LD 2 the second group – 1 mg/kg of the active ingredient, or 0. 4 ml/kg (concentration of active ingredient in the drug was 2. 4 mg/ml), corresponding to 1/100 of the LD_{50}. Control animals, under the same conditions and feeding were administered an equal volume of a solution of excipients included in the formulation without the active ingredient at the rate of 0. 8 ml/kg of the drug with no active ingredient.

During the experiment monitored the condition and behavior of animals, the dynamics of growth of body weight, regularly conducted studies to assess the functional state of the liver, kidney, and studied the effect of the drug on hematology. Statistical processing was performed by the Student-Fischer.

The results showed that in the experience of external signs of toxicity in animals were reported. All animals as experimental and control groups were active, the hair shiny, smoothed. Response to external stimuli is preserved.

No signs of toxicity and death of animals was observed, which gives rise to an absence of the drug in doses of cumulative effect on the toxic principle.

Traditional Husbandary Practices and Major Health Problems of Camels Ion Egypt

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Abstract: The present study was undertaken to provide information about cutaneous abscesses, wounds and sores of camels and occurance of pneumonia in them. Also, the role played by drinking animals from contaminated artesian wells was considered.

The total number of camels included was 50 of different ages, sex, and belonged to Matrouh governorate, Egypt. Of these animals, 19 showed respiratory signs, 20 dead and 11 slaughtered. 31 cutaneous abscesses, wounds and sores were observed on the humps and feet-pads of most animals.

For bacteriological examination, samples were aseptically collected from nasal discharge of diseased animals(19). Lung abscesses of dead and slaughtered cases (20 and 11 respectively), humps and feet-pads abscesses, wounds and sores (14 and 17 respectively). Also, 16 samples from drinking artesian wells for animals were aseptically collected.

Culture of samples, isolation and identification of causative agents were carried out according to the well known methods.

The test of antibiogram was applied on most isolated strains using the disc diffusion technique of antibiotics.

Anorexia, coughing, muco-purulant nasal discharges, respiratory distress and elevated body temperature (39 – 40℃) were the prominent clinical signs, of the diseased camels.

A number of pathogenic bacteria including *Proteus* spp. (12), *K. pneumoniae* (12), *E. coli*(20), *Staph. aureus* (16), *Strept.* spp. (12) *P. aeruginosa*(8) and the yeast *Candida albicans*(16) were isolated from the various samples. Equal incidence of 12.37% was shown by *Proteus* spp., *K. pneumoniae* and *Strept.* spp. The most important isolated bacteria was *E. coli*(21.65%). The isolated *Staph. aureus* has the same incidence of *Candida albicans*(16.49%). The lowest incidence was recorded in *P. aeruginosa*(8.25%).

The antibiogram pattern showed various sensitivity percentages among the isolated microorganisms. Some strains of *Proteus* spp., *K. pneumoniae*, *E. coli* and *P. aeruginosa* showed resistance to Ampicillin. 100% resistance of *P. aeroginosa* strains to Ampicillin, Cefacitrile, Chloeamphenicol, Gentamycin and Penicillin was reported. Kanamycin and Streptomycin showed various degrees of sensitivity among all bacterial isolated. Most of the isolated *Candida albicans* seemed to be resistant to most of antibiotics discs.

Only 5 strains (31.3%) of isolated *Candida albicans* were sensitive to Penicillin.

It could be concluded that saddles which are not well fitted and loaded and improperly balance well injure the humps of camels. Also walking camels in the desert will hurt its feet-pads from pointed edges of some stones and the prickles of some plants.

All these damaged cutaneous lesions may be contaminated from the environmental pathogens. Also, drinking of contaminated water with pathogens should be considered internal or external environmental pollution of air, water and earth in desert areas with pathogens will cause health problems in camels.

Kanamycin was seen to be the drug of choice for health problems in camels.

Molecular Typing and Biofilm Formation of *Escherichia coli* O157 in Eastern China

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Abstract: *Escherichia coli* O157 is food-borne pathogen that cause diseases in human, which has become a major public health concern worldwide. Food contaminated with *E. coli* O157 has been identified as a potential source of transmission of these pathogen in human. Thus, this study was performed to detect and characterize *E. coli* O157 strains isolated from animal feces and food samples in Eastern China, which will help to understand the extent of food contaminated by animal feces. *E. coli* O157 was isolated and detected in 2.08% animal fecal samples (18/864) and 1.03% food samples (2/194), indicating that *E. coli* O157 was more prevalent in animal fecal than foods. Virulence genes and molecular typing of all isolates were determined, which showed that *E. coli* O157 in food and fecal samples were clustered into same predominant group. This result indicated that animal feces might be a reservoir for *E. coli* O157. Thus, close monitoring of the possible contamination of food by animal feces should be reinforced. The biofilm formation assays showed that 40% of the *E. coli* O157 could produce biofilm. However, composite analysis of all three molecular typing methods to biofilm formation revealed biofilm formation of *E. coli* O157 was independent of molecular genetic typing used in this study.

Insight into the Specific Virulence Related Genes and Toxin-Antitoxin Virulent Pathogenicity Islands in Swine Streptococcosis Pathogen *Streptococcus equi* ssp. *zooepidemicus* Strain ATCC35246

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Abstract: **Background**: *S. zooepidemicus* is the ancestor of *Streptococcus equi* ssp. *equi* (*S. equi*) and they expresses many of the same proteins and virulence factors, but unlike the *S. equi*, which is host-restricted and only infects horse, *S. zooepidemicus* has no host preference. This bacterium is primarily an opportunistic pathogen infecting a wide variety of animal species, including important domestic species, so it is a pathogen of veterinary concern. In China, it is an important pathogen of swine streptococcosis, causing septicemia, meningitis, endocarditis and arthritis, resulting in significant economic losses. Occasionally, it can infect humans via zoonotic transmission from the infected animals and cause invasive infections in humans such as septicemia and meningitis. In 1975, Sichuan Province experienced a *S. zooepidemicus* pandemic, resulting in the death of 300,000 pigs and great economic losses. To date, it remains a great threat to the chinese swine breeding. Pathogenicity islands (PAIs) of *S. zooepidemicus* were transferred among bacteria through horizontal gene transfer (HGT) and played important roles in the rapid adaptation and increased virulence of *S. zooepidemicus*.

Results: Genome of *S. zooepidemicus* ATCC35246 (Sz35246) is 2,167,264-bp and composed of a single circular chromosome with GC content of 41.65%. We made the X-alignment analysis of Sz35246 versus *S. zooepidemicus* MGCS10565 (Sz10565), *Streptococcus equi.* ssp. *equi.* (*Streptococcus equi.*) 4047 (Se4047) and *S. zooepidemicus* H70 (Sz70), 320 genes were identified as specific to the Sz35246 genome, these genes shaped the evolution of the Sz35246 genome and some of them might make this bacteria able to cause swine streptococcosis. Genes led *S. zooepidemicus* to break through the host-restriction were identified too. The transcriptomes of bacteria *in vivo* and *in vitro* revealed many genes might be related to Sz35246 infection. Through the analysis between *S. zooepidemicus* and *Streptococcus equi.*, genes of CRISPR/Cas system and Fim Ⅲ operon were presumed to be involved in the broking through of host-restricted of Sz35246. The genome contains four acquired pathogenicity islands (PAIs) encoding proteins which potentially contribute to the overall pathogenic capacity including infecting pig and fitness of this microbe. Sequence and annotation analyses of these islands reveals that SeseCisland_1&SeseCisland_2&SeseCisland_3 contain the same kind of known virulence genes involved in bacterial toxin-antitoxin (TA) systems which have been reported to play an subtle roles in survival of the bacterium under harsh natural environments. These three TA systems are related to DNA replication, mRNA stability, protein synthesis, cell-wall biosynthesis and ATP synthesis, including Phd/Doc TA system, Fic/Doc TA system and ε/ζ TA system. Otherwise, the restriction modification system (RM system) is found in SeseCisland_4 and used by bacterials to protect themselves from foreign DNA, such as bacteriophages and other viruses. After comparing Sz35246, Sz70, Sz10565 and Se4047, we found some of the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-associated genes (SeseC_00664, SeseC_00667-00669 and SeseC_00671-00673), including Cas1, Cas2, Cas4b and Cas5, only existed in *S. zooepidemicus* genome. CRISPR/Cas system may provide acquired immunity to the bacteria against viruses and plasmids, some Cas proteins are involved in the acquisition of novel spacers, others provide CRISPR-encoded phage resistance and interfere with invasive genetic elements. Some genes, including *mal*A (SeseC_01626), *mal*D (SeseC_01627), *mal*E (SeseC_01633, SeseC_01622), *mal*F (SeseC_01624, SeseC_01630), *mal*G (SeseC_01625), *mal*Q (SeseC_01617) were up-regulated when Sz35246 infecting mouse. These genes are related to maltose transport and metabolism, utilization of carbohydrates is essential to the ability of pathogenic bacteria to cause disease. We also found some well-known virulence factors up-regulated when Sz35246 infecting, for example, streptokinase (SeseC_02411) and fibronectin-binding protein (*sfs*, SeseC_00464). The up-regulated of bacteriocin (SeseC_02042) will help Sz35246 compete with other bacteria colonized in host and make sure this pathogenic bacteria gain enough live space and easier to invade host.

Conclusion: The genome and expression analysis of Sz35246 provides basic information on the physiology and potential pathogenic capacity of this bacterium. The comparison of the genomes of Sz35246, Se4047, Sz10565 and Sz70 provides the specific genes of Sz35246, these genes may be related to the pathogenic function including cause swine streptococcosis and breaking host-restriction. We found some new mobile genetic elements, which are involved in the evolution of Sz35246. These elements and the phylogenetic analysis indicate that this genome is shaped by chromosomal inversion, recombination and HGT events. Sz35246 acquired its PAIs and some specific genes through HGT. TA system exists in 3 genomic islands of Sz35246; this system participated in many biological activities, including pathogenicity. We will pay more attention on that in our further research. Ultimately, the availability of the complete Sz35246 genome sequence will facilitate further studies of this pathogen and the development of diagnostics and vaccines.

Zoonotic Disease and Prevention

Transmission in Guinea Pigs of Airborne H9N2 Avian Influenza Viruses Isolated from Chicken Houses

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Summary: This study aimed to determine the transmission characteristics of H9N2 avian influenza viruses (AIVs) derived from the air. Eight H9N2 AIVs were isolated from chicken houses between 2009 and 2010. We analyzed the phylogenic and pathogenic traits of these isolates. What's more, transmission characteristics in guinea pigs of two airborne isolates were determined in experimental conditions. Phylogenetic analyses indicated that the homologies of HA and NA genes of eight isolates were 95.4% – 99.7% and 86.6% –99.8% respectively. They were able to duplicate in lung tissues of guinea pigs without prior adaptation. Two airborne isolates could both transmit among guinea pigs by direct contact. No infection was detected in aerosol contact animals while H9N2 AIV aerosols were detected in the air of isolators. In conclusion, H9N2 AIV aerosols were detected in the environment of mammals, suggesting that urgent attention will need to be paid to its transmission.

Introduction

H9N2 avian influenza virus (AIV) has spread in many countries and areas since first surfacing in America in 1966. Although its pathogenicity was trivial, it could cause high morbidity and lethality to poultry when co-infected with other pathogens (Bano et al., 2003; Haghighat-Jahromi et al., 2008; Homme and Easterday, 1970; Nili and Asasi, 2002). In mainland China, after firstly being isolated from diseased chickens in 1994, H9N2 AIV began to spread widely and clustered in the Eurasian lineage (Choi et al., 2004; Li et al., 2005; Xu et al., 2007).

Notably, H9N2 AIVs have lent urgency to their pathogenicity to humans. From 1999 to 2003, H9N2 influenza viruses were detected from nasopharyngeal aspirates of the children with influenza-like illness in Hong Kong (Butt et al., 2005; Peiris et al., 1999). Recent studies have suggested that an amino acid mutation Q226L in hemagglutinin (HA) gene providing H9N2 AIV with the ability of binding to the SA α2-6 receptor, which enabled its replication and infection in mammalian hosts (Matrosovich et al., 2001; Wan et al., 2008). Previous studies demonstrated that H9N2 AIVs could transmit among domestic poultry through aerosols (Shi et al., 2010; Yao et al., 2011). Once acquiring the capacity of airborne transmission in human beings, they would become a great threat to public health. No airborne transmission of wild H9N2 AIV has been reported in mammals (Wan et al., 2008). In present study, we reported an H9N2 AIV sublineage spreading in eastern China with replication ability in guinea pigs. The data demonstrated that H9N2 AIV could transmit by direct contact in guinea pigs and viral aerosols could be detected in the isolator.

Material and methods

Virus

H9N2 AIV strains were isolated from tracheal and cloacal swab samples of diseased chickens and air samples of 11 chicken houses in Shandong province, China, according to the method reported previously (Lv et al., 2011). Based on the source of samples and gene sequences analysis, eight representative viruses were selected (Table 1).

Sequencing and phylogenetic analysis

Viral RNA was extracted from the allantoic fluids using the TIANamp Virus RNA Kit (Tiangen Biotech Co. Ltd., Beijing, China) following the manufacturer's instructions. The RT-PCR process followed the protocol reported previously with minor modifications (Hoffmann et al., 2001; Lu et al., 2005). Nucleotide sequences and amino acid sequences were edited and aligned using DNASTAR software (Madison, WI, USA), and phylogenetic trees were produced using Clustal X (Version1.81) and MEGA 4.0 software with neighboring-joining method.

Pathogenic analysis

Some 4-week-old SPF chickens inoculated intranasally (i.n.) with 10^6 EID_{50} of each virus did not behave any clinical symptoms in preliminary experiments. So chickens were inoculated i.n. with high dose 10^7 EID_{50} and observed the behavior, clinical symptoms, feed intake and lethality for 21 days.

In regard to pathogenicity to mammals, groups of five hartley strain female guinea pigs weighing 300 g and serologically negative for influenza virus were anesthetized with ketamine (20 mg/kg) and xylazine (1 mg/kg), and inoculated intranasally (i. n.) with 10^6 EID_{50} of infectious allantoic fluid to investigate the replication of the virus (animals were died with $10^7 EID_{50}$ inoculation in preliminary experiments). Three animals of each group were euthanized on 5 days post inoculation (dpi) and clarified homogenates of lungs were titrated for virus infectivity in MDCK cells from initial dilutions of 1∶10 (lung) as previously described (Lu et al., 1999). The remaining two animals were observed for two weeks for signs of disease and death, and their sera were collected on 14 dpi. Seroconversion was confirmed by HI assay.

Experimental transmission in guinea pigs

To assess the viral transmission in mammals, two isolates (ACSDA and ACSDM) from air and chicken swab samples simultaneously were used in two independent experiments. Briefly, three anesthetized guinea pigs were inoculated i. n. with the virus (10^7 EID_{50} of ACSDA or 10^6 EID_{50} of ACSDM) and housed in a cage in isolator A. Twenty-four hours later, three naive animals (direct contact) were introduced into the same cage, meanwhile three animals (aerosol contact) were placed in another cage that was 80 cm away. Another three (aerosol contact) were placed in isolator B, which was connected a tube (length: 1 m; diameter: 0.08 m) to allow air to flow from A to B. Nasal washes were collected at 2 day intervals and titrated in MDCK cells. Seroconversion was confirmed by HI assay on 14 dpi.

Air samples were collected simultaneously using an AGI-30 liquid sampler (Brachman et al., 1964; Lv et al., 2011). The sampler, containing 20 mL of phosphate buffered solution (PBS, 0.1 M, pH 7.2) with 1000 U/mL of penicillin and 1 mg/mL of streptomycin, was operated continuously for an optimized time of 30 min (t) at an airflow rate of 12.5 L/min. After ultracentrifuged, A 0.292-mL sample of air was removed to extract RNAs for real-time RT-PCR which was performed on a standard 7500 Real-Time PCR System, using methods reported previously, to quantify the airborne AIVs (Lv et al., 2011).

Results and discussion

Phylogenetic analysis and molecular characterization

Phylogenetic analysis of the HA gene showed that eight isolates shared 95.4% – 99.7% similarity and belonged to HKY28097-like lineage. For NA gene, they showed 86.6% – 99.8% similarity and ACSDM was identified as belonging to HKG997-like lineage, while the others belonged to BJ194-like lineage. All viruses were in the sublineage h9.4.2 and the lineage n2.1 in a new panorama phylogenetic diversity (Liu et al., 2009). Amino acid analysis showed that the sequences at the cleavage site between HA1 and HA2 were ^{335}RSSR^{338}, ^{335}GSSR^{338} and ^{335}KSSR^{338} which demonstrated that they were H9N2 LPAI viruses (Kawaoka and Webster, 1988; Steinhauer, 1999).

Pathogenicity of H9N2 AIV

Results demonstrated that all viruses were not lethal to chickens while lethal to guinea pigs at the inoculation dose of 10^7 EID_{50}. Guinea pigs showed inactivity, inappetence, visible signs of labored respirations and sneezing on 2 – 6 dpi. Some animals became emaciated and died on 4 – 6 dpi, but survived animals began to recover clinically after 7 – 8 dpi. The viruses could replicate efficiently to high titers in the lungs of guinea pigs and cause seroconversion with HI titers of 40 – 80. Virus titer of lungs infected by ACSDM at the dose of 10^6 EID_{50} was the highest, while ACSDA infected lungs was the lowest which was significantly lower than most virus (Table 1). The eight isolates with Leu226 residue could be infectious to mammals. Because of their widely spreading in poultry, we designed experiments to study if they may transmit among mammals by aerosol.

Transmission of H9N2 AIV

ACSDA and ACSDM isolates were selected to examine the transmission ability among guinea pigs (Fig. 1). In two independent experiments, virus was detected in nasal washes of three inoculated guinea pigs between 2 – 6 dpi. In ACSDM experiment, viruses were isolated from the nasal washes of three direct contact guinea pigs between 4 – 8 dpi. For ACSDA experiment, viruses were isolated from only one direct contact guinea pig. Seroconversion occurred in all inoculated groups on 14 dpi with anti-H9 antibody titers of 40 – 80. They were in the range of 10 – 20 for direct contact guinea pigs. No viral shedding or seroconversion was detected in any aerosol contact guinea pigs.

Air samples collected by AGI-30 liquid sampler were analyzed using the 7500 System SDS Software Version 1.2 (Table 2). Viruses were detected in ACSDM experiment on 6 dpi, reaching its peak on 8 dpi (average concentration 7.91×10^4 copies/m^3) and dropping to 6.28×10^4 copies/m^3 on 10 dpi; no viral aerosols were detected on 12 dpi. Many studies had revealed that influenza virus had the ability to transmit by aerosol among mammals such as guinea pigs and ferrets and also presented in the emergency department air (Blachere et al., 2009; Munster et al., 2009; Van Hoeven et al., 2009). H9N2 AIV can also spread in poultry by means of aerosols (Shi et al., 2010; Yao et al., 2011). No animal infected in our aerosol group might correlate with many factors, such as the infective dose, aerosol particle size, and aerosol particle half-life time or viability of

virus, etc (Tellier, 2009). Previously studies found that some gene structures of the virus were important factor for virus aerosol transmission. H9N2 AIV with a human H3N2 backbone can transmit efficiently via respiratory droplets (Sorrell et al., 2009). Studies discovered that PB2 amino acids 627 and 701 are determinants of mammalian inter-host transmission in H5N1 AIV and human H3N2 backgrounds (Steel et al., 2009). To better understand the H9N2 AIV transmitting among mammals, further studies about other gene structures are also needed.

Conclusions

H9N2 AIV aerosols could be detected in the environment and may infect mammals at certain concentrations, so it is critical to sterilize environment to decrease virus aerosol concentration and block their transmission.

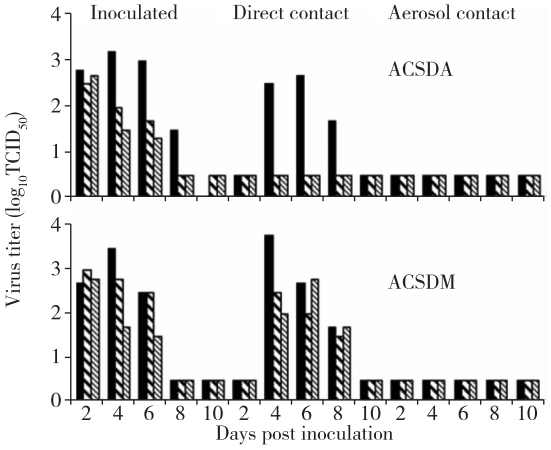

Fig. 1 Transmission of H9N2 AIV in guinea pigs. Each bar represents the virus titers of every animal nasal washes. Virus titers were not detected from nasal washes of aerosol contact animals in isolator B, so they were not represented in this figure.

Table 1 Background information and the pathogenicity in animals of H9N2 AIV isolates

Virus	Abbreviation	Samples [a]	Replication in guinea pigs [b]		Lethality [c]		GenBank accession No. [e]
			Virus titers in lung (log_{10}TCID$_{50}$/gram ± SD)	Seroconversion (HI titers)	Chickens	Guinea pigs	
A/Chicken/Shandong/A/2009	ACSDA	Swabs and air	2.6 ± 0.2 * (3/3) [d]	80, 80	0/3 [d]	1/3	HQ225839, HQ225838
A/Chicken/Shandong/B/2009	ACSDB	Swabs	3.4 ± 0.4 * (3/3)	40, 80	0/3	1/3	JN683642, JN683648
A/Chicken/Shandong/C/2009	ACSDC	Swabs	3.6 ± 0.2 * (3/3)	80, 80	0/3	1/3	HQ378727, HQ378728
A/Chicken/Shandong/E/2010	ACSDE	Swabs	3.3 ± 0.5(3/3)	80, 80	0/3	0/3	JN683643, JN683649
A/Chicken/Shandong/K/2010	ACSDK	Swabs	3.4 ± 0.6 * (3/3)	80, 80	0/3	0/3	JN683644, JN683650
A/Chicken/Shandong/L/2010	ACSDL	Swabs	3.2 ± 0.5(3/3)	40, 80	0/3	0/3	JN683645, JN683651
A/Chicken/Shandong/M/2010	ACSDM	Swabs and air	3.8 ± 0.2 * (3/3)	80, 80	0/3	1/3	JN683646, JN683652
A/Chicken/Shandong/N/2010	ACSDN	Swabs	3.4 ± 0.4 * (3/3)	80, 80	0/3	0/3	JN683647, JN683653

[a] Viruses were isolated from chicken tracheal and cloacal swabs and air samples collected by AGI-30 liquid sampler. (Lv et al, 2011).
[b] Guinea pigs (n = 5) were infected i. n. with 10^6 EID_{50} of each virus in a 300 μL volume.
[c] SPF chickens (n = 3) and guinea pigs (n = 3) were inoculated i. n. with 10^7 EID_{50} of each virus and observed the animals clinical signs for 21 days.
[d] The number of positive/total number tested for each virus.
[e] The numbers refer to HA and NA genes of H9N2 AIV.

Table 2 Mean concentration of airborne H9N2 AIV in isolator

dpi	Real-time RT-PCR [a] (copies/reaction)		Airborne H9 AIVs concentration [a] (copies/m^3 air)		Infective dose (copies)
	ACSDA experiment	ACSDM experiment	ACSDA experiment	ACSDM experiment	ACSDM experiment
2	–	–	ND	ND	ND
4	–	–	ND	ND	ND
6	–	3.68×10^2	ND	4.20×10^4	2.72×10^4
8	–	6.93×10^2	ND	7.91×10^4	5.13×10^4
10	–	5.50×10^2	ND	6.28×10^4	4.07×10^4
12	–	–	ND	ND	ND

[a] Two samplers were used for one isolator, and the liquid was pooled as one sample.
–, No virus was detected from the air samples on this day; ND, not done.

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Outbreak of Mastitis Caused by Strains of Methicillin-Resistant *Staphylococcus aureus* (MRSA) in a Closed Dairy Herd

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Summary: Mastitis caused by *Staphylococcus aureus* (*S. aureus*) is one of the main diseases in dairy herds. This is an important pathogen due to a combination of toxin-mediated virulence, invasiveness, and antibiotic resistance. Cows are probably the main source of contamination of raw milk with *S. aureus*. In particular, cows with subclinical mastitis can shed large numbers of *S. aureus* in their milk. The epidemiology of *S. aureus* has changed radically in particular, methicillin-resistant *S. aureus* (MRSA), originally restricted to hospital, has emerged as a significant pathogen in the community, and true community-acquired MRSA (CA-MRSA). Considering the potential risk to the public health as well as to animal health, the aim of this study was to verify an outbreak of mastitis caused by *S. aureus* in one dairy farm and were analyzed the presence of the *mecA* gene. Milk samples were obtained from all mammary glands of all lactating cows. The isolates were identified by conventional methods. To confirm the identification of *S. aureus* we used the primers Staur 4 e Staur 6 previously described. Polimerase Chain Reaction was used to determine the presence of *mecA* gene using primers previously described. A total of 103 cows was analyzed, of the 407 mammary glands screened with CMT, 115 were positive and from those, *Staphylococcus* spp. were isolated from 61 (59%). Of these isolates, 60 (98.4%) were *S. aureus* and 1 (1.6%) were *S. epidermidis*. It was detected the presence of gene *mecA* in 48.3% *S. aureus* strains. The detection of this large number of MRSA was a great concern in the animal health, considering that beta-lactams are still the most important antimicrobials in the treatment of mastitis. Besides that *Staphylococcus aureus* isolates from bovine mastitis represent a potential hazard to public health.

Introduction

Mastitis caused by *Staphylococcus aureus* (*S. aureus*) is one of the main diseases in dairy herds. *S. aureus* is an important pathogen due to a combination of toxin-mediated virulence, invasiveness, and antibiotic resistance. This bacterium is a significant cause of nosocomial infections, as well as community-acquired diseases.

Cows are probably the main source of contamination of raw milk with *S. aureus* [1]. In particular, cows with subclinical *S. aureus* mastitis can shed large numbers of *S. aureus* in their milk. The epidemiology of *S. aureus* has changed radically in particular, methicillin-resistant *S. aureus* (MRSA), originally restricted to hospital, has emerged as a significant pathogen in the community, and true community-acquired MRSA (CA-MRSA). Considering the potential risk to the public health as well as to animal health, the aim of this study was to verify an outbreak of mastitis caused by *S. aureus* in one dairy farm and was analyzed the presence of the *mecA* gene.

Material and methods

Milk samples were obtained from all mammary glands of all lactating cows were screened using the California Mastitis Test (CMT) and a strip cup [2]. The isolates were identified by conventional methods. To confirm the identification of *S. aureus* we used the primers Staur 4 e Staur 6 previously described by Straub et al. (1999) [3]. Polimerase Chain Reaction (PCR) was used to determine the presence of *mecA* gene. Extraction of DNA was carried out using Illustra Blood GenomicPrep Mini Spin Kit (GE Healthcare, Bukinghamshire, UK). PCR reactions were performed using primers previously described by Murakami et al. (1991) [4]. The amplification was performed in a DNA thermal cycler (Mastercycler, ep eppendorf, Hamburg, Germany), and the amplicons were visualized by electrophoresis in 2% agarose gel with SYBR Safe DNA gel stain (Invitrogen, California, USA), in concentration of 1 μL in 10 mL of gel. *S. aureus* (ATCC 33591 and ATCC 25923) were used as a positive control.

Results

A total of 103 cows was analyzed, of the 407 mammary glands screened with CMT, 115 were positive, from which milk samples were collected and used for microbiological examination. Of the 115 CMT-positive milk samples, *Staphylococcus* spp. were isolated from 61 (59%). Of these isolates, 60 (98.4%) were *S. aureus* and 1 (1.6%) were *S. epidermidis*.

It was detected the presence of gene *mecA* in 48.3% *S. aureus* strains these results were presented in Table 1.

Table 1 Gene *mecA* detection on bovine mastitis *S. aureus* isolates. Botucatu-SP. 2012.

Staphylococcus aureus	No.	%
mecA positive	29	48.3
mecA negative	31	51.7
Total of *S. aureus* isolates	60	100.0

Conclusions

The detection of this large number of MRSA were a great concern in the animal health, considering that beta-lactams are still the most important antimicrobials in the treatment of mastitis. Besides that *Staphylococcus aureus* isolates from bovine mastitis represent a potential hazard to public health.

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Endosymbionts in Ticks of Norway—A Possible Target of Biological Manipulation and Disease Control?

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Summary: Ticks have an almost global distribution and tick-borne diseases are of growing concern with regards to both animal and human health. Examples of pathogenic bacteria transmitted by ticks are: *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, *Francisella tularensis*, *Rickettsia helvetica*, *Bartonella* spp., *Babesia* spp. and tick-borne encephalitis (TBE) virus. Changes in climatic conditions have been suggested to affect the spatial distribution of ticks, but other factors such as growing wild game populations, migratory birds and grazing conditions also play an important role. About 80% of the world cattle population is exposed to ticks, which leaves ticks to be the ecto-parasite of greatest economic impact on livestock productions worldwide.

About 300,000 lambs are infected annually by the tick-bacterium *A. phagocytophilum* (tick-borne fever) in Norway. The disease results in great economic losses to the farming communities and large animal welfare challenges. Recently, there has been an increasing focus on biological methods for the control of tick populations and diseases as a result of increasing antimicrobial resistance attained by parasites and microbes. Tick pathogens have received much attention, but ticks do also carry non-pathogenic organisms that are endosymbionts. One theory is that endosymbionts previously were disease-causing micro-organisms in animals that, in the course of evolution, were acquired by ticks when taking blood meals. These endosymbionts may become important in the biological control of ticks and their diseases in the future. A pilot project was initiated in 2010. The aim was to investigate whether the newly discovered symbiont *Midichloria mitochondrii* occurs in ticks (*Ixodes ricinus*) in Norway. Three areas in Rogaland and Hordaland counties were selected as sampling sites as these are regions where ticks and tick-related disease are highly prevalent.

Introduction

Midichloria mitochondrii was first described in 2004 [2]. This intracellular bacterium has been discovered to inhabit oocytes and other ovarian cells. It is found not only in the cytoplasm but also in the cell mitochondria, which may indicate that it interferes with the energy metabolism and reproduction of the tick [8]. Unlike *A. phagocytophilum*, *Midichloria* is transmitted vertically between generations of ticks (I. ricinus). DNA fragments of Midichloria and other closely related bacteria have previously been isolated in mammals and recent studies have revealed the association of this organism with tick salivary glands [6]. The same study found that the serological prevalence of *Midichloria* infection in human patients presented with tick bites was 58% (n = 80). In healthy blood donors, the prevalence was only 1.2% (n = 169) [6]. These findings may lead to questions like; does M. mitochondrii have a vital biological function in the tick (reproduction or metabolism) and is the bacterium capable of being transmitted to a mammal through blood feeding and between ticks by co-feeding? The project aimed to investigate whether symbionts, and especially *M. mitochondrii* could be found in ticks in Norway. Blood samples were also collected from sheep and wild ruminants in the sampling areas to investigate whether these were infected by the same species of symbionts as were found in ticks.

Material and methods

From three distinct geographical locations, 76 blood samples from sheep, 532 ticks and 14 tissue samples (12 from red deer, 2 from sheep) were collected in the field. Tissue samples from deer were taken during the hunting season in the autumn of 2010 in cooperation with local hunters. The samples were analyzed for the presence of *Borrelia* spp., *Anaplasma phagocytophilum*, *Rickettsia* spp., *Wolbachia pipientis* and *Midicloria mitochondrii* by real-time PCR amplification of regions in the bacterial DNA. Specific products were identified by melting point analysis against known standards. Larvae and nymphs were analyzed in "pools" of five and adults as individuals [4].

Results

Wolbachia sp. was not detected. However, *M. mitochondrii* was detected in various tick developmental stages from three different areas in Hordaland and Rogaland counties. However, the relative frequency of *M. mitochondrii* was higher in larvae and nymphs than in adults. The nymphs showed particularly high infection rates *of M. mitochondrii*. The number of positive samples (total) from each site were; Tysvaer 53/93, Etne 17/42, Sandnes14/17. Thirteen of 84 positive tick samples contained two or three agents (co-infected). Among these there were one tick (pooled nymphs) containing *Borrelia*, *Anaplasma* and *Midichloria* (7.70%), eight ticks (nymphs only) containing *Borrelia* and *Midichloria* (61.54%), two ticks (adults) containing *Anaplasma* and *Midichloria* (15.38%) and two ticks (one adult and one pooled nymphs) containing both *Rickettsia* and *Midichloria* (15.38%). Among the samples containing only single agents (71/84), one tick (pooled nymphs) contained only *Borrelia* (1.40%), two ticks (one pooled nymphs and one adult) contained only *Rickettsia* (2.82%), two ticks (one pooled nymphs and one adult) contained only *Anaplasma* (2.82%) and 66 (four pooled larvae, 45 pooled nymphs and 17 adults) contained only *Midichloria* (92.96%). No symbionts were detected in the animal samples however *A. phagocytophilum* was present in 56% of them.

Discussion

Previous studies have shown that nearly 100% of female ticks are carriers of *M. mitochondrii* [5] and less than half of males (44%) [8]. In the present study, the ticks were not separated by gender and *M. mitochondrii* was found in 100% of larval samples, 60.44% of nymph samples and 35.09% of adult ticks. The results indicate that only some of the adults seem to be carriers of *M. mitochondrii*, which may mean that the involvement of the endosymbiont in tick reproduction is less significant than previously thought [9]. On the other hand, this remains only tentative since individuals were not sexed. However, it is interesting that all larval samples (only represented by 2.63% of the samples and 3.76% of the individuals) and a large proportion of the nymphs are positive for *M. mitochondrii*. If this reflects reality, it may indicate that *M. mitochondrii* is an important part of the early development stages (larva and nymph). Whether all larvae and nymphs are infected, needs to be investigated in future work, since they were pooled in this study. It should also be investigated what happens in the different developmental stages if *M. mitochondrii* is removed from the tick. Will any of the physiological processes such as reproduction and metabolism change? Furthermore, it is desirable to investigate whether this endosymbiosis is involved in the transmission of pathogens as no combinations of two or more infectious agents were found without *Midichloria* being present. Based on the results from this and other studies, a joint international research project on the importance of endosymbionts should be conducted. It is important to study which endosymbionts have a vital biological function in ticks and their interaction with host animals. Previous studies have indicated that symbionts may play an important role in tick physiology and affect population dynamics and influence the transmission of pathogenic rickettsiae [1]. Endosymbionts have been shown to cause changes in fertility in a number of insects by provoking early embryo death, "male killing," shifted sex ratio (parthenogenesis: haploid eggs develop into living females), feminization (sex change from male to female) and unsuccessful mating between infected males and uninfected females or individuals infected with different strains of symbionts (cytoplasmic incompatibility). The latter can result in sterile males or lack of offspring [3, 11]. This incompatibility can be of great evolutionary importance ("speciation") and will be very important for the manipulation of tick populations by the introduction of symbionts. This biological approach may also be used to reduce animal and public health problems associated with ticks in the future [7]. Furthermore, it will be important to consider whether humans and animals are carriers of endosymbionts [10].

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Pathological Classification of Tuberculosis Lesions in Positive Intradermal Tuberculin Test Cattle in Taiwan

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Summary: Implementation of test and slaughter policy to control and eradicate bovine tuberculosis (TB) has been undertaken in Taiwan for the past 65 years. The prevalence of the disease was around 0.13% to 0.27% in total of one hundred thousand dairy cattle raised in the last 5 years. In order to pursuit a better TB control management in this area, it is necessary to obtain a contemporary data of pathology profile in tuberculin reactors. From 2007 to 2008, 57 positive intradermal tuberculin test (ITT) cattle were collected from 4 ITT positive farms in central Taiwan. Post-mortem investigation was performed at the rendering plants and 4 lymph nodes (LNs) including retropharyngeal, mediastinal, tracheobronchial, and mesentery lymph nodes as well as lung tissue were collected and were subsequently preceded for microbiological culture, histopathological examination, lesion scoring, and acid fast special stain. The results showed that visible TB-like lesions were recorded in 27 of 57 ITT positive cattle (47.4%) and single lesion (20/27) was predominantly present. Retropharyngeal LN was the most common affected site (22.8% or 13/57) and the mean severity score (MSS) was the highest (0.46), compared to mediastinal lymph node (14.0%; MSS 0.25), tracheobronchial lymph node (12.3%; MSS 0.16) and mesenteric lymph node (10.5%; MSS 0.18). Lung lesions were only observed in one animal. Acid-fast bacilli were very difficult to find (2/27) and *Mycobacterium bovis* were isolated in 12 of 27 positive TB-like lesions cases. Finally, 13 of 27 positive TB-like lesions animal were less than 1 year old and the MSS of calf was 0.93 which was different from 1.38 in adult animal. In addition, the lesions in young animal were more located at the mesentery LN.

Introduction

Bovine tuberculosis (TB) is an important zoonotic disease which is chiefly caused by an acid-fast bacillus *Mycobacterium bovis*. The disease has severe economic impact leads to enormous economic lose on agricultural manufacture around the world. Implementation of test and slaughter policy by intradermal tuberculin test (ITT) has been carried out to control bovine TB since 1956 in Taiwan. This management has successfully reduced the prevalence of bovine TB from the beginning of 8.57% to the last five years of 0.13% – 0.27% [1]. In ITT-negative dairy farms, every individual animal which is over 1 year old should have ITT annually. However, in ITT-positive dairy, ITT is performed every 3 months. If these farms could sustain 3 consecutively negative results of ITT, they could be resumed as ITT-negative farms. Under this intensive monitoring of bovine TB; however, low prevalence of ITT-positive animal or ITT-positive dairy farms still remains in this area.

The total number of dairy cattle raised in Taiwan is about one hundred thousand heads; however, these animals are distributing in 642 dairy farms. Comparing to the developed country, the scale of the dairy farms in Taiwan is relative small and their density is relative high. In addition to those environment conditions that may enhance the transmission of the pathogen of bovine TB, two important reasons may explain why the disease has not been eradicated in this island. These may include lack of the investigation system to further examine the tuberculin reactors and no feedback system for granulomatous lesions found in the abattoir.

The aims of the present study is to obtain a contemporary data of pathology profile in tuberculin reactors by post-mortem investigation, bacterial isolation, histopathological examination, lesion scoring, and acid fast special staining.

Material and methods

This study was conducted in Yunlin County, which is located at the central western area of Taiwan and the county fosters a total of 12,000 dairy cattle, during the period of October 2007 to October 2008. Fifty-seven cattle displaying positive reactions of ITT were collected in this study at the rendering plants before bone-meal manufacturing. All the sampled cattle were dairy cows and heifers. The age of collected cattle were categorized into 2 groups as adult (greater than 1-year-old; n = 28) and young (less than 1-year-old; n = 29). With the exception of one cattle was apparent emaciated, almost all (n = 56) cattle show normal body condition on appearance.

Gross lesions were detected and recorded attention focus on the retropharyngeal lymph node, hilar lymph node, mediastinal lymph node, mesenteric lymph node and bilateral lung because of the highest possibility of tuberculous lesion presence of which of being reported in previous studies [4]. Lung lobe was incised for 2-cm-thick intervals and each lymph node was medial section to facilitate the detection of visible lesion on the cut surface grossly. Typical lesion suggestive of tubercles includes white to light yellowish foci on the lung or lymph node consisting of firm nodules and military parenchymae. Necrotic and abscesseous changes may present in the central of involved areas. A part of each sample was simultaneously collected within sealed plastic bags to reserved at ice bucket for further bacterial isolation and culturing.

All the specimens collected were processing for histopathological examination. Tissue fixed in 10% neutral formalin over 24 hours was trimmed for routinely paraffin-embedment and was sectioned and stained with hematoxylin and eosin for histopathological examination. Interpretation of lesion suggestive of tuberculosis was based on the presence of granulomatous inflammation with or without central caseous necrosis and mineralization. Duplicate sections were manufactured and stained with Ziehl-Neelsen methods to identify the acid-fast bacilli in tuberculous lesions

The severity scoring criteria used in the present study was modified from previous studies in order to adapt the availability [4]. Each sample including lung, retropharyngeal lymph node, hilar lymph node, mediastinal lymph node, and mesenteric lymph node was scored individually based on a semiquantitive system as follow: 0 = there is no gross and microscopic lesion suggestive of tuberculosis; 1 = there is no visible tubercle lesion on gross but lesion apparent on microscopic examination; 2 = the lesion is present on both macroscopic and microscopic examination for local or few areas; 3 = both macroscopic and microscopic examination show extensive necrosis or multiple abscesses accompanied with severe granulomatous inflammation. A total pathology score for each individual was obtained by the sum of all severity scores from lung and lymph nodes. The scoring process was performed by the same inspector to assure the scoring operation consistency.

Lesions thought to be associated with bovine tuberculosis on alternative gross or histological examination were referred to collaborated laboratory for mycobacterial culture. Each submitted specimens were cultured on Lowenstein-Jesen media for 12 weeks period to observe the bacterial growing

Results and discussion

All the 57 collected cattle underwent post-mortem examination for the detection of gross tuberculous lesion. Sixteen of 57 cattle showed visible lesions at single or multiple sites in varying degree that were determined to be tuberculosis. Extremely enlargement, multiple nodular bulging accompanied with central necrostic abscesses in lymph node were the most predominant detectable characteristics on appearance. A total of 27 of 57 cattle revealed microscopical lesion suggestive of tuberculosis that includes all the cattle having gross lesions ($n = 16$) and 11 cattle without showing significant tuberculous lesions on gross but being identified on histological examination. Multiple clusters of aggregations of activated macrophages resembling as "epithelioid cells" were randomly dispersed among the lymph nodes regardless of which sites. Low to moderate numbers of multinucleated giant cells may admix within the granulomas. Central caseous necorsis consisted of massive cellular debris with or without mineralization. Ziehl-Neelen staining for the identification of acid-fast bacilli was performed on tuberculosis occurring on histopathological slides. Only 2 of 27 cattle showed rare number of acid-fast bacilli as only one or two bacillus in the cytoplasm of multinucleated giant cell on the slides.

Most of lesion-involved cattle (20/27; 74.1%) possessed tuberculous lesion at single site predominantly rather than multiple distributions. The comparison of frequencies of lesion occurrence among retropharyngeal lymph node, hilar lymph node, mediastinal lymph node, mesenteric lymph node and lung. Retropharyngeal lymph node showed the highest frequency of tuberculous lesion presence (22.8%; $n = 13$), followed by mediastinal lymph node (14.0%; $n = 8$), hilar lymph node (12.3%; $n = 7$), and mesenteric lymph node (10.5%; $n = 6$). Only 1 cattle (1.8%; $n = 57$) contained tuberculous lesion at pulmonary lobes extensive involved adjacent pleural tissue forming multiple tubercle nodules. The most severely affected lymph node was retropharyngeal lymph node (mean pathology score was 0.46), followed by mediastinal lymph node (as mean of 0.25), mesenteric lymph node (as mean of 0.18), and hilar lymph node (as mean of 0.16).

Of the aged diversity, tuberculous lesions were found in 14 adult cattle (50%; $n = 28$), compared to 13 of young cattle (44.8%; $n = 29$) were affected. However, the frequency of tuberculous lesion did not differ ($\chi^2 = 0.16$, $P > 0.05$) between adult and young cattle on statistical analysis by chi-square (χ^2) test. The distribution of lymph nodes with gross discernible lesion in adult cattle was significant different ($\chi^2 = 8.74$, $P < 0.05$) that was most frequently present in retropharyngeal

lymph node (5/9). In contrast, there was no difference of lesion tropism in young cattle ($\chi^2 = 2.10$, $P = 0.55$). The mean severity score of adult cattle (greater than 1-year-old) was higher than young cattle's (less than 1-year-old), of 1.38 and 0.93, respectively. The comparison of pathology severity of mesenteric lymph node is noteworthy between adult and young cattle; in adult cattle, merely two of them (7.1%) contained tuberculous lesions in mesenteric lymph nodes, additionally, both the severity degree were defined as 1 (mean score was 0.08 among 28 adult cattle); in contrast, more frequently (13.8%) and more severely (two of score 3, one of score 2 and one of score 1; mean score was 0.31 among 29 young cattle) tuberculous lesions in mesenteric lymph nodes of young cattle were found.

The frozen-preserved tissues with tuberculous lesions were referred to collaborated laboratory for bacterial culture. The growth of *Mycobacterium bovis* took place in 13 of 27 cattle whose tissues were properly processed through 12 weeks long period of culture.

Although ITT is performed annually in each individual dairy cattle in Taiwan, low prevalence and sporadic incidence of ITT reactors are still present in this area. In the present study, TB-like lesion were present in 47.4% of ITT-reactors. The distribution and severiety of the TB-like lesions may be similar or different from other countries [2,3,4], the lesions may be not induced or exposured by high dosage of bacteria [5]. Significant porportion of ITT reactors were young animal and predominent lesions were found in the mesenteric lymph node suggests earlier infection possible through oral route may be occurred [6]. In summsry, the present study suggests ITT need to perform earlier on young cattle and continuing pathological examination in ITT reactor is necessary.

Conclusions

Although ITT is performed annually in each individual dairy cattle in Taiwan, low prevalence and sporadic incidence of ITT reactors are still present in this area. The distribution and severiety of the TB-like lesions may be similar or different from other countries, significant proportion of ITT reactors were young animal and predominent lesions were found in the mesenteric lymph node suggests earlier infection possible through oral route may be occurred. The present study suggests ITT needs to perform earlier on young cattle and continuing pathological examination in ITT reactor is necessary in this region.

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Investigation of Thermophilic *Campylobacter* Prevalence in Organic Turkey Farms in Germany

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Summary: The demand for organic food of animal origin has increased considerably in recent years. The widespread assumption that the free-range environment contaminates extensively reared turkeys has not been rigorously tested. The aim of this study is to provide current information about the prevalence of thermophilic *Campylobacter* in five organic meat producing turkey farms in different regions in Germany as well as on genotypes and possible sources of contamination. The clinical and environmental samples were identified as *Campylobacter* by conventional and molecular methods. The isolates were differentiated and typed by *flaA*-RFLP analysis.

Campylobacter spp. were detected in caecal swabs in all 5 turkey farms with prevalence (90% to 100%). Both *C. jejuni* and *C. coli* were detected with prevalence ranged from (14% to 67%) and (33% to 86%), respectively. *Campylobacter* was detected in drinkers and in dark beetles. No *Campylobacter* was isolated from main water tanks. *flaA*-RFLP assay showed that turkey farms can harbour more than one genotype. In a single turkey two different types could be detected.

Observations of this study revealed that organic turkey flocks are often contaminated already at an early age with *Campylobacter*, drinking water and also dark beetle can play a role in the entry of *Campylobacter* into turkey flocks, a high degree of similarity between *C. jejuni* genotypes from both farm sites, higher genetic differentiation between *Campylobacter* populations from turkeys. A more thorough understanding of the transmission pathways is necessary in order to fight the spread of *Campylobacter* in organic turkey flocks by biosecurity control measures.

Introduction

The demand for organic food of animal origin has increased considerably in recent years. Important parts of the organic farming philosophy are that animals should have access to outdoor areas, which can result in increased risks of food safety problems. In addition birds should be slaughtered as close to the production site as possible, thereby avoiding the stress that long distance transportation may incur [1].

In broilers and other meat producing poultry organic production may exert a major influence on carrier rates. This poses a risk that they possible get infected with *Campylobacter*.

A marginally higher prevalence of *Campylobacter* spp. was observed in organic rather than in conventional laying hen husbandries in Germany [2].

The prevalence of *Campylobacter* was significantly higher in organically raised broilers than in conventionally-raised broilers, while the prevalence of this organism in organically-raised turkeys was not significantly different from that in conventionally-raised turkeys [3].

In general, the conventional turkey farms harbor more antibiotic-resistant *Campylobacter* strains than organic poultry farms [3].

Biosecurity measures can reduce *Campylobacter* shedding rates of housed poultry, but the increasing popularity of free-range organic meat raises the question of whether the welfare benefits of extensive production are compatible with food safety.

Colonization of free-range poultry, with *Campylobacter* from their environment is widely thought to be a likely route of infection [4].

However, the high diversity and wide distribution of *Campylobacter* populations in wild and farm animals has seriously complicated the investigation of infection [4]. Conversely, the presence of a genotype in the

environment does not in itself prove a source of contamination or route of transmission [4].

This aim of this study was to assess the prevalence and extent to which young turkeys reared under organic conditions become infected with campylobacters.

Material and methods

Sampling of turkey farms

Samples were collected from 5 different free-range meat turkey flocks. The flock sizes ranged from 1000 to 2000 birds (Kelly BBB or B. U. T. 6) per flock. The birds were about 4 to 8 weeks old. Cloacal swabs were taken from 30 randomly selected birds at each flock, streaked separately on mCCDA (Oxoid) and then transported to the laboratory for incubation. Drinking water (main water tank and drinker) and beetles were also investigated. Temperature and relative humidity (RH) were also measured during sampling.

Isolation and identification of *Campylobacter*

Isolation was performed in accordance with the ISO 10272-1 (2006) [5] guideline. Pure cultures were obtained by cultivation on Columbia blood agar (Oxoid, Germany) and then 5 suspected colonies were identified phenotypically. Initially positive isolates were further identified using the biochemical reaction profiles obtained by the API Campy System (BioMerieux, Germany) according to the instructions of the manufacturer.

Chromosomal DNA extraction

Genomic DNA was extracted from a 48 h bacterial culture on MH blood agar plates using High Pure PCR Template Preparation Kits (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The DNA was eluted in 200 μl elution buffer. DNA was quantified spectrophotometrically using a Nanodrop® ND-1000 (Fisher Scientific GmbH, Schwerte, Germany).

Species confirmation and *flaA*-RFLP assays

The identified isolates were confirmed as *C. jejuni* by using a multiplex PCR (mPCR) assay as described previously [6]. For *flaA*-RFLP analysis extracted DNA was amplified, as described elsewhere [7]. The *flaA* amplicon was digested for 18 h at 37℃ with *Dde*I (Roche Diagnostics GmbH). The DNA segments were separated using 2.5% agarose gels (Starlab GmbH, Hamburg, Germany) in TBE buffer at 200 V for 1 h, stained with ethidium bromide and visualized under UV light. Documentation was done using a Bio Imaging System (Syngene, Cambridge, UK).

Results and discussion

The organic system performed better for the economic indicator net farm income per Full Time Equivalent, than the conventional system [8]. Although the organic system performed better than the conventional system for animal welfare and less antibiotic residues in meat but more *Campylobacter* contaminations than meat from a conventional system.

It is found that *Campylobacter* is highly prevalent (90% to 100%), in all 5 organic turkey production systems (Table 1) that in agreement with the previous studies [3, 2]. The *Campylobacter* isolates were identified as *C. jejuni* and *C. coli*. The rapid movement of a *Campylobacter* infection through a poultry house and its flock may be facilitated by feed and water supplies used [9].

Drinking water may play role in transmission of *Campylobacter* to turkey farm [6]. In this study *C. jejuni* was detected in drinkers of 2 turkey farms with the same genotype with the isolate from the cecal sample (Fig. 1). Moreover dark beetles of the 2nd farm were positive for *Campylobacter*. No *Campylobacter* was isolated from main water tanks. *flaA*-RFLP assay showed that turkey farms can harbour more than one genotype in one production cycle (4 types of *C. coli* in flock 4, 5 and 5 of *C. jejuni* in flock 5) as discussed before [7]. The surprising results we can isolate two different genotypes from a single turkey.

Table 1 The within flock prevalence and genotypes of *Campylobacter* isolated from 5 organic turkey farms

	Flock 1	**Flock** 2	**Flock** 3	**Flock** 4	**Flock** 5
Prevalence of *C. jejuni*	26.67%	63.33%	14.29%	56.67%	66.67%
Prevalence of *C. coli*	73.33%	26.67%	85.71%	63.33%	33.33%
Drinking water samples	positive	positive	negative	negative	negative
Dark beetles sample	negative	positive	negative	negative	negative
C. jejuni genotypes	3	4	1	2	5
C. coli genotypes	2	1	2	4	4

Conclusions

In conclusion, *Campylobacter* contamination being the main risk factor for organic turkeys, the specific risk factors for *Campylobacter* contamination on organic farms need further study.

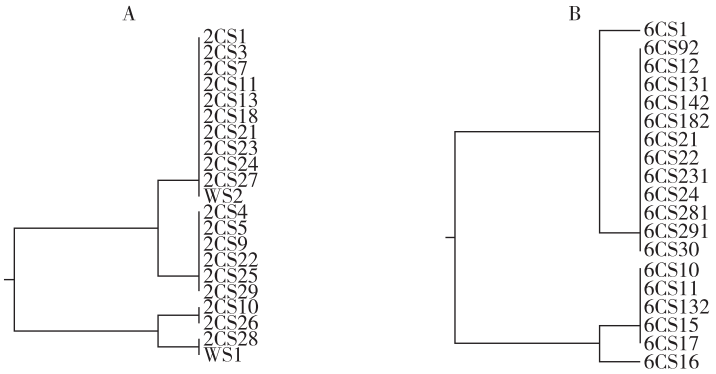

Fig. 1 Dendogram of restriction profiles of 21 *C. jejuni* - *flaA* gene (A) and 19 *C. coli* - *flaA* gene isolates (B) were characterized into 4 different genotypes using restriction endonuclease *Dde*I.

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Mycobacterium bovis Genotypes in Central Area of Taiwan

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Summary: The campaign against bovine tuberculosis (TB) has been carried out in Taiwan based on a principle of test-and-slaughter program for more than 50 years. However; sporadic outbreaks contributed to a fluctuant prevalence of tuberculin test reactors still remained. In order to pursuit a better TB control management in this area, it is necessary to obtain a contemporary status of bovine TB in tuberculin reactors. A total of 57 intradermal tuberculin test (ITT)-positive dairy cattle originated from 4 individual farms in a central-western county in Taiwan, were collected and sampled for pathologic investigation, bacterial isolation, molecular diagnosis and genotyping. TB-like lesions were identified in 27 cattle (47. 4%; n = 57) with the visible gross and/or microscopic lesions. *Mycobacterium bovis* (*M. bovis*) were identified in 24 out of 27 positive TB-like lesions cattle (88. 9%) by PCR and *M. bovis* were isolated in 12 of them. The variable number tandem repeat (VNTR) method based on six particular targets, as named as extracted tandem repeats loci, was used to proceed molecular genotyping. Three genotypes of *M. bovis* could be obtained from the samples of 15 positive TB-like lesions cattle. The findings uncovered the status of bovine TB in ITT-positive cattle in Taiwan. The VNTR is applicable to distinguish variant strains of *M bovis* and would be helpful for further epidemiology study and eradication program.

Introduction

A national bovine tuberculosis (TB) eradication program in dairy cattle based on annually single intradermal tuberculin test associated with slaughter program for skin test reactors was initiated in Taiwan in 1956. At the first decade, the prevalence of tuberculin reactors dramatically decreased from 8. 57% in year 1950s to 0. 76% in year 1960s. Between 1970 and 1996, the percentage of skin test-positive cattle intermittently wax and wane in the range of 0. 02% to 0. 75%. At the latest decade, the prevalence of tuberculin reactors maintained around 0. 05% and failed to reduce any more.

In Taiwan, bovine skin test is applied four times a year in the TB positive farm. To prevent the spread of the zoonotic pathogens in the field, recent policy suggests the skin test-positive cattle are euthanized and send to rendering plant directly without any pathological or microbiological examination. It is important to investigate the skin test reactors, not only to understand the severity of the lesions but also to confirm the pathogens among attested cattle. Re-confirming the pathogens among tuberculous-like lesions is necessary because not only *Mycobacterium bovis* but also other closely-related mycobacterial species enable to cause similar tuberculous-like lesion and elicit positive skin test reaction (Lindeboom et al., 2006; Monaghan et al., 1994)[1].

The aim of this study is to investigate the pathology of skin test reactors at Yun-Lin county in Taiwan, and further microbiological identification and phylogenic analysis to determine the bacteria species in the lesions by PCR and VNTR respectively were also included.

Material and methods

This study was conducted in Yunlin County which locates in central-western part of Taiwan. The area of this county is about 1300 km^2 and the population are around 3 quarter million. The total number of dairy cattle in this county was estimated to be 12,000 which are dispersing in 70 individual self-employed farms. Between October 2007 to October 2008, a total of 57 cattle from 4 individual farms (A-D) in Yunlin, were determined as positive skin test reactors. Farm A had 31 skin test reactors collected from the results of five series skin tests. Farm B and farm C had 14 and 6 skin test reactors, respectively, and both were collected after one time screen. Farm D had 6 skin test reactors after two consecutive screens. All of the 57 cattle were positive in intradermal tuberculin test and were undergone detailed postmortem examination in the rendering plant and retropharygeal lymph node, hilar lymph node, mediastinal lymph node, mesenteric lymph node and lung were collected.

All collected specimens were separated at field for preparation of histopathologic and molecular examination. Of which the tubercle-like lesion presenting on gross, the lesion area was bisected into equivalents for both evaluations. Each half section of specimen for

histopathogic examination was sliced less than 1-cm-thick and fixed in a solution contained 10% neutral buffered formalin fixative. All other haft compartments prepared for molecular evaluation were packaged in sealed plastic bags and placed in an ice bucket immediately. All specimens collected from field were processing for histopathologic investigation. Tissue fixed in 10% neutral formalin over 24 hours was sliced into 5-mm-thickness and processed by routinely paraffin-embedment. Each paraffin sections was cut at 3-micrometer thick and stained with hematoxylin and eosin for detailed histopathologic examination. Interpretation of lesion suggestive of tuberculosis was based on the presence of granulomatous inflammation with or without central caseous necrosis and mineralization. If tubercle-like lesion was observed, duplicate sections were stained with Ziehl-Neelsen methods for identification of acid-fast bacilli among the lesion. When tuberculous-like lesion was identified on pathologic examination, the lesion-involved specimens were referred to collaborated laboratory for mycobacterial culture. Each submitted samples were cultured on Lowenstein-Jesen media for up to 12 weeks and inspected weekly for colonies.

Tissue lysis and DNA purification is performed using DNeasy® Blood & Tissue kit (Qiagen Inc., CA.) followed by routine manufacture's introduction. Purified DNA was stored at −20℃ until test be continued. Three major PCR tests in this study, which consisted of amplification of partial 16*S rRNA* gene conserved among all mycobacterial species and closely bacterial genus (Roth and others 1998) [2], *TbD1* gene for *Mycobacterium bovis*, and *RD8* gene for *Mycobacterium tuberculosis* (Brosch and others 2002) [3], were conducted in parallel for all collected specimens whether including the tubercle lesion or not. The PCR condition and procedure were followed as described in previous two studies.

Fifteen isolates from 15 of the 57 cattle were genotyped. The condition and procedure of VNTR method based on six particular targets, as named as extracted tandem repeats loci, was followed in reported literature (Frothingham and Meeker-O'Connell, 1998) [4]. These loci vary in number of internal tandem units that gives the length of alleles in variable size. The size of sequence fragments were estimated under UV light exposure, and then transferred to number code by the formula for each locus unit. Genetic diversity of different strains was demonstrated by comparison of six VNTR numbers in variant sort. The discriminatory power of VNTR genotyping was assessed by the allele diversity (h) of individual and combined VNTR loci using the following mathematic equation: $h = 1 - \sum\chi_i^2 [n/(n-1)]$, where χ_i is the frequency of ith allele at individual locus, and n is the number of assessed isolates.

Results and discussion

The tuberculous lesions were detected in total 27 cattle (47.4%; 27/57), of which of 14 cattle possessed tuberculous lesions on microscopic examination, exclusively, but not show any visible lesion on the gross.

All the DNA samples extracted from each lymph node and lung among 57 cattle were performed by PCR assessments for identification of mycobacterial species. Twenty-nine of assessed cattle (50.9%; 29/57) were positive for amplification. Of the cattle with lesion resembling tuberculosis, 66.7% (18/27) of them were 16S rDNA amplified positive. On the other hand, 11 (36.7%) of cattle without any tuberculous lesion in either lymph nodes or lung were PCR positive. The result of amplification of 16S rRNA was consistent with lesion presence ($P < 0.05$, $\chi^2 = 5.18$).

For specific detection of *Mycobacterium bovis*, the *TbD1* gene amplifications were carried out on all extracted DNA samples regardless of the amplified results of 16S rRNA. The overall rate of *Mycobacterium bovis* detection among 57 skin test positive cattle was determined as 47.3% (27/57) based on this PCR test. In contrast to 16S rDNA, most of TbD1 positive cattle were relevant to the presence of pathologic lesions suggestive of tuberculosis as shown as 24 of 27 (88.9%) lesion-involved cattle were determined for *Mycobacterium bovis* reservoirs. Moreover, three of assessed cattle without any tuberculous lesion on pathologic examination were tested to be positive by TbD1 amplification assay. Predominant consistency was shown between the TbD1 amplification and lesion involving ($P > 0.01$, $\chi^2 = 35.47$). None of the specimen in this study was positive for amplification of *RD8* gene where is specific to *Mycobacterium tuberculosis*.

The frozen-preserved tissues with tuberculous lesions were referred to collaborated laboratory for bacterial culture. The growth of *Mycobacterium bovis* took place in 13 of 27 cattle whose tissues were properly processed through 12 weeks long period of culture. All the cattle with positive culture of *Mycobacterium bovis* revealed positive PCR amplification for *TbD1* gene.

Fifteen of 27 *Mycobacterium bovis* isolates determined by PCR amplification were performed on VNTR typing using six polymorphic loci (ETR-A to -F). A total of three different genotypes were identified among 15 isolates based on polymorphism of alleles. The discriminatory power differed for the individual locus, which displayed variable allele diversity (h) ranging from 0.00 to 0.53. Among the six VNTR loci (ETR-A to -F), ETR-B showed the highest discriminability that differentiated three allelic types and the allele diversity (h) was 0.53. The next three loci, ETR-A, ETR-C and ETR-E,

constituted similar allele diversity (h) for 0.44. There was no allele diversity ($h = 0.00$) for ETR-F representing monomorphic allele among 15 isolates. The combination of all six loci gave high allele diversity (h) as 0.75.

Seven *Mycobacterium bovis* isolates from farm A collected by various date showed similar VNTR phynotype that was classified as genotype I. There were two different strains of *Mycobacterium bovis* isolates according to variant VNTR phynotypes defined as genotypes II and genotypes III simultaneously present in farm B. The isolate from farm C was evaluated to be genotype II as similar as isolates from farm B. In the present study, six ETR-VNTR loci (ETR-A to F) were used in strain typing of *Mycobacterium bovis* isolates. A high discriminatory power ($h = 0.75$) was displayed by combination of these 6 VNTR loci among 15 *Mycobacterium bovis* isolates. Among the 6 sets of VNTR loci, ETR-B showed the highest polymorphism ($h = 0.53$), followed by ETR-A, ETR-C and ETR-E also showed high allele diversity ($h = 0.44$ for each), and both ETR-D and ETR-F performed a low discriminatory capacity ($h = 0.07$ and 0.00, respectively) among the 15 assessed isolates. It suggests that the low discriminatory loci, ETR-F in particular, can probably be deleted from this VNTR analysis system in further large-scale investigation. Not all cattle confirmed as *Mycobacterium bovis* infection were successfully analyzed by VNTR method because of the failures of amplification and/or exhibition of discernible bands on electropherograms. The plausible reason is presumably similar with the limitation of 16S rRNA amplification as above described.

Overall, VNTR typing discriminated three different genotypes dispersing in farm A, farm B and farm C in this study. Genotype I, detected from seven cattle, was exclusively responsible for the multiple outbreaks of bovine tuberculosis in farm A during the period from October 2007 to July 2008. It was indicative of an important epidemiological information that similar strain of *Mycobacterium bovis* did not eradicated by repetitive culling and remained in a persistent infection. Two *Mycobacterium bovis* genotypes (II and III) co-existed at once outbreak in farm B. The existence of genotype III was also found at another outbreak of bovine tuberculosis in farm C. Interesting, it is a short distance, merely one km, between farm B and farm C, and the outbreaks took place on close time (less than 40 days).

Conclusions

In summary, the prevalence and infection condition of bovine tuberculosis in Taiwan is very consistent with those reported in development countries based on pathologic investigation. PCR is a useful tool to ensure the true burden of whether the pathogenic mycobacteria infection or not. The application of molecular genotyping is valuable on epidemiological studies providing important information for national tuberculosis eradication program in the future.

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LA-MRSA Inside and in the Vicinity of Turkey and Broiler Farms: A Longitudinal Study

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Summary: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a well-known pathogen occurring in human as well as in veterinary medicine. Until now little is known about transmission pathways of MRSA in poultry farms as well as about the emission of MRSA from poultry houses. We investigated the occurrence of livestock associated (LA-) MRSA inside and outside of previously MRSA positive screened poultry farms in Germany. Therefore, five turkey and two broiler fattening farms were investigated four respectively three times during the fattening period with the focus on airborne transmission. Samples of animals like swabs of choana and skin, the animals' environment inside the barn including air samples as well as samples of the barns' vicinity like ambient air and boot swabs of the ground in different distances up to 500 m to the barn were taken. LA-MRSA was found in the stable air in six out of seven barns as well as in the ambient air outside of two turkey barns. The ground surfaces on the downwind side were significantly more frequently tested positive for LA-MRSA (44.4%) with detections up to a distance of 500 m than these on the upwind side (26.9%). The same *spa* types of MRSA origination from samples of inside and outside could be observed which leads to the assumption of an emission of MRSA from the farms in their surrounding. Interestingly, no detection of MRSA was possible in any sample of broilers or their environment inside the barn at the first samplings shortly after arriving of the animals in the two investigated farms. Later the resistant microorganism could be detected in different samples. Perhaps MRSA is not mainly introduced to the broiler farms via colonized chicken hatchlings. In conclusion, a transmission of MRSA within poultry farms as well as outside of the farms via the airborne route seems to be one possible way.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a well-known pathogen occurring in human as well as in veterinary medicine. It was first described as hospital acquired MRSA in nosocomial infections [1], later as community acquired MRSA in healthy humans without hospitalisation [2]. In 2005 MRSA was found in healthy pigs [3] and many studies about these so called livestock associated (LA-)MRSA especially in pigs [4] but also in cattle, calves and poultry followed. In one of our previous studies, MRSA was found in the ambient air as well as on soil surfaces around pig farms [5]. The objective of this study was to estimate the prevalence of MRSA in poultry farms and to analyse the distribution of MRSA on positive farms inside and outside the barns over time.

Material and methods

Only poultry farms which had been tested positive for MRSA previously or at the beginning of a production cycle were included in the studies. Therefore, 85 turkey fatting farms and 40 broiler fattening farms were tested by investigating boot swabs and in most cases also dust as well as swabs of skin and choana.

Five turkey farms and two broiler farms were analysed four respectively three times during the fattening period with samples taken from animals (60 choana and skin swab samples), the barn s interior (air samples by impingement and filtration, dust, faeces, feed, boot swab) and exterior (ambient air by impingement and filtration, boot swabs of the ground). Broiler farms were investigated for the first time shortly after arrival of the hatchlings on fattening day 2, turkey farms within the first nine weeks after arrival.

Spa typing was performed on 80 MRSA isolates of different samples from all farms (results not shown).

Results and discussion

25.9% (22/85) of previously screened turkey fattening farms and 22.5% (9/40) of the fattening broiler farms were tested MRSA positive.

The results of the longitudinal study are summarized in Table 1.

They show that LA-MRSA can be found inside and in the vicinity of poultry farms [6]. MRSA was found at 13 out of 26 sampling dates in barn air samples and was

not detected in exhaust air samples of the upwind side of all barns. In air samples from the downwind side, MRSA was detected in five samples with very low concentrations due to a strong dilution of these microorganisms after leaving the barn (7 – 93 cfu/m^3). MRSA was found in 44.4% (36/81) of all environmental boot swab samples taken from the surfaces of the downwind side (including the samples of all distances, two MRSA positive swabs in 500 m distance not shown in Table 1), compared to 26.9% (7/26) on the upwind side. As the same spa types of isolates were detected inside and outside of the barns an emission of MRSA from the barns can be assumed. However, an additional emission of microorganisms from the surrounding barns or by fertilized farm land is possible [7].

Interestingly, in both investigated broiler farms all tested chickens as well as all other samples inside the barn were negative for MRSA one day after arrival of the hatchlings in the barn. This may indicate that MRSA is not mainly introduced to the farms via colonized chicken hatchlings.

The relevance of the spread of MRSA from poultry houses to their environs is not clear. However, a transmission of MRSA via the air or contaminated surfaces is generally possible and local residents and neighbouring livestock might be exposed. Although MRSA concentrations in ambient air was very low making a direct airborne colonization of animals and people housed or living close to poultry farms unlikely. This topic including the sedimentation of bacteria in the surrounding needs to be studied in more detail.

Table 1 MRSA detection inside and in the vicinity of five turkey fatting farms (Ⅰ – Ⅴ) and two broiler fattening farms (Ⅵ, Ⅶ) [6]

Farm No.	Downwind from the investigated barn																				Inside the investigated barn												Upwind from the investigated barn							
	soil 300 m				soil 150 m				soil 50 m				air 150 m				air 50 m				dust				air				choana				air 100 m				soil 100 m			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Ⅰ	–	–	–	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	–	–	+	–	+	+	–	–	–	–	–	–	–	–
Ⅱ	–	+	–	–	–	+	+	+	–	+	+	+	–	–	–	–	–	–	–	–	+	+	+	–	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	–
Ⅲ	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	o	–	–	–	o	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
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1 – 4 stand for the different time points of sampling during one fattening period from the beginning to the end; turkey farms were sampled four times and broiler farms three times; + = MRSA positive sample; – = MRSA negative sample; o = no sample was taken in this interval

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Isolation and Detection of *Streptococcus gallolyticus* subsp. *gallolyticus* from Organic Turkey Flocks

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Summary: *Streptococcus gallolyticus* subsp. *gallolyticus* can be an opportunistic pathogen in animals and humans. Its role as a potential zoonotic pathogen is discussed. However, very little is known about the occurrence of this bacterium in turkey flocks. Thus we developed a selective isolation method for culturable *S. gallolyticus* subsp. *gallolyticus* poultry faeces. First isolation tests were conducted with pigeon faeces from different holdings and the results showed that the tannase activity of *S. gallolyticus* subsp. *gallolyticus* and its possibility to grow in the presence of sodium azide were successful characteristics to select the bacterium from streaks on solid media. Isolates were identified to species level by biochemical tests and to subspecies level by MALDI-TOF mass spectroscopy and *sodA* sequencing. Further laboratory tests demonstrated that the application of the detection method is also useful for the isolation from turkey faeces. In a field study we investigated thirteen organic turkey flocks from ten different farms in Germany. Five fresh droppings were taken from each flock and analyzed within 24 h in the laboratory. *Streptococcus gallolyticus* subsp. *gallolyticus* occurred in all sampled flocks. The prevalence in positive droppings was 0. 83. To the best of our knowledge, this is the first investigation that indicates a widely spread of *S. gallolyticus* subsp. *gallolyticus* in organic turkey flocks. The developed detection method seems to be a suitable tool to detect transmission ways and may contribute to the investigation of the zoonotic potential of *S. gallolyticus* subsp. *gallolyticus* in future studies.

Introduction

Streptococcus gallolyticus subsp. *gallolyticus* (formerly *S. bovis* biotype I) occurs in the gut of humans and animals as a commensal but can also be an opportunist or an infectious agent. This subspecies can cause e. g. streptococcosis in pigeons or in young turkeys associated with an increased mortality risk [1]. Furthermore, several studies showed a significant coincidence between endocarditis and colorectal cancer in humans [2]. However, the knowledge about transmission ways and the zoonotic potential of *S. gallolyticus* subsp. *gallolyticus* is currently unclear [3]. Although the molecular biological methods to compare the genetic relationship between human and animal isolates are available, studies about the dissemination and prevalence of this bacterium in farm animals are still missing. One reason therefore is probably the difficulty to isolate this subspecies from the normal gut flora. The aim of this study was to develop a suitable method to detect culturable *S. gallolyticus* subsp. *gallolyticus* bacteria from farm animals and to use this method for the isolation and identification of this bacterium in organic turkey flocks.

Material and methods

The isolation and cultivation of *S. gallolyticus* subsp. *gallolyticus* was developed in the laboratory by using fresh droppings of pigeon faeces. The use of a solid medium (CASO agar, OXOID LTD, Basingstoke, Hampshire, England) modified by adding tannin and sodium azide allowed the separation of *S. gallolyticus* subsp. *gallolyticus* from the bacteria flora in faeces when incubated for 48 h at 37℃ in a CO_2 atmosphere. Therefore 2 g of faeces were dissolved in 4 ml PBS buffer and stored for 30 min. at room temperature. Subsequently the sample was shaken on a vortex and a loop of the suspension was streaked out on the modified CASO agar. After incubation a part of a suspected colony was streaked out on Columbia agar plates (CASO agar, OXOID LTD, Basingstoke, Hampshire, England) with 5% sheep blood. The inoculated plates were incubated at 37℃ for 24 to 36 hours. Isolates were identified by morphological characteristics, basic biochemical reactions (e. g. catalase and oxidase reaction) and by microscopy before a pure culture was analysed by MALDI-TOF mass spectroscopy and *sodA* sequence analyses as described by Hinse et al. [4]. This method was used to detect *S. gallolyticus* subsp. *gallolyticus* in fresh droppings from 13 organic turkey flocks of 10 different poultry farms located

in the north, in the east or in the middle part of Germany. Birds age ranged between four and 18 weeks. Five randomly selected fresh droppings were sampled from each flock and analysed within 24 h as described above.

Results and discussion

S. gallolyticus subsp. *gallolyticus* was found in all investigated flocks. The ages of the flocks were four, six, eight, nine, eleven, 15, 16, 17 or 18 weeks. The bacterium was found 11 times in five out of five droppings, one time in three out of five droppings and one time in one out of five droppings. The overall prevalence was 0.84 in 65 fresh droppings indicating a high prevalence of *S. gallolyticus* subsp. *gallolyticus* among turkeys in organic flocks. No increased mortality occurred in the investigated flocks.

It is known that *S. gallolyticus* subsp. *gallolyticus* can be a causative agent in broiler and turkey flocks and an infection can increase the mortality within a flock [1, 5]. However, studies about the prevalence of this bacterium in poultry flocks are missing and it is unknown which risk factors lead to an infection in poultry flocks. The reason therefore may be the less importance of economic losses due to *S. gallolyticus* subsp. *gallolyticus* infections. On the other hand the impact of these infections could be underestimated because the isolation and identification raise difficulties [5]. It is supposed that the isolation and identification method described in this report is useful to conduct further prevalence studies in farm animals. This may also give the opportunity to learn more about the zoonotic potential of *S. gallolyticus* subsp. *gallolyticus*. The adaption of *S. gallolyticus* subsp. *gallolyticus* strains to different species and the transmission from animals to humans is discussed controversy. Meanwhile the developed method of this study was also tested successfully to isolate culturable *S. gallolyticus* subsp. *gallolyticus* from human faeces (data not shown). Therefore it is assumed that the effective isolation of *S. gallolyticus* subsp. *gallolyticus* with a modified CASO agar enables comprehensive epidemiological studies about its zoonotic potential in future.

Conclusion

An effective isolation method for cultivating *S. gallolyticus* subsp. *gallolyticus* from farm animals and probably also from human faeces was developed. The application of this method can very likely contribute to assess the zoonotic potential of this bacterium in future studies.

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Zoonoses Denouement on Public Health and Economy of India: An Overview

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Summary: Zoonotic infections pose a significant public health challenge for low and middle income countries and have traditionally been a neglected area of research. India ranked topped in the list of countries worst affected by zoonotic diseases (those originating from animals). Livestock is an important sub sector of Indian agriculture contributing 6.71 billion dollar per annum accounting for 25% of the output of agricultural sector and about 22.45 million people work in livestock sector. In India livestock are important in supporting livelihood of poor farmers, consumers traders and labourers throughout the country. Close association between human population and animals, consumption of unpasteurized milk and dairy and inappropriate carcass disposal are some of the principle factors perpetuating infection in humans. The scope of this paper is restricted to the diseases present in India. The public health importance of these diseases are also emphasized. The clinical aspects, transmission, disease course, diagnosis and control of such diseases are discussed. Guidelines are presented for the inspection of the animal origin food for human consumption. The presence of Zoonotic diseases can be detected by observation of clinical signs ante mortem & by detection of localized or disseminated lesions in the carcass & viscera post mortem. Abnormal behaviour can indicate Rabies, Listeriosis, Tetanus or FMD. Skin lesions can result from TB, Paratuberculosis, Anthrax. Diarrhoea can suggest Salmonellosis, Colibacillosis etc. Based on epidemiological data of active surveillance programme it is estimated that due to Brucellosis there is loss of US $ 58.5 million per year and FMD causes $ 5 billion revenue loss in India. Therefore, necessary preventive measures are required to control these zoonotic diseases.

Key words: anthrax, brucellosis, cysticercosis, India, leptospirosis rabies, salmonellosis, zoonotic diseases, zoonotic tuberculosis

Diseases and infections that are naturally transmitted between vertebrate animals and humans have been defined as Zoonoses. 60% of all infectious disease pathogens and 75% of all emerging pathogens falls in the category of zoonotic diseases. (WHO, 2010; Woolhouse and Gaunt, 2007). Zoonotic diseases are of great public health importance in India (Chengappa et al, 2007) which can be explained by the fact that livestock is an important sub sector of Indian agriculture contributing 6.71 billion dollar per annum accounting for 25% of the output of agricultural sector and 68% of the workforce relies on farming that is in close contact with domestic animals and poultry with frequent exposure to sick or infected animals. According to Sehgal and Bhatia (1990), intimate and prolong contact between man and animal facilitates the transmission of various communicable zoonotic diseases between man and animals.

According to study conducted by International Livestock Research Institute (ILRI, 2012) India is among the top geographical hotspots for zoonotic diseases followed by Ethiopia, Nigeria and Tanzania. With the world's second largest human population, two biodiversity hotspots (Myers et al, 2000), and one of the world's greatest densities of tropical livestock (Thornton et al, 2002), India possesses a favourable environment for the transmission of communicable diseases between man and animals (Jones et al, 2008; Forman et al, 2008). Other factors responsible for dessiminating zoonotic diseases are: unhygienic living conditions, lack of education, poor personal hygiene, poverty and occupation. Wildlife also served as an important reservoir of zoonoses either infecting humans directly or indirectly through domestic animals. Furthermore, several relevant zoonotic infections may be vector borne, that is, transferred from animals to humans via, for example, arthropods or ticks. Many zoonotic infections actually are promoted by human behaviour such as bush-meat hunting (EBOLA fever), the farming and trade of live wild animals (SARS), close and repeated contacts with infected animals (avian influenza), deforestation, which brings humans closer to infected vectors and animal reservoirs (leishmaniasis), or building of dams, that favours the proliferation of mosquitoes (Rift Valley fever). All major zoonotic diseases prevent the efficient production of food of animal origin, particularly of much-needed proteins, and create obstacles to international trade in animals and animal products. They are thus an impediment to overall socioeconomic development in India. The presence of Zoonotic diseases can be detected by observation of clinical signs ante mortem & by detection of localized or disseminated lesions in the carcass & viscera post

mortem. Abnormal behaviour can indicate Rabies, Listeriosis, Tetanus or FMD. Skin lesions can result from TB, Paratuberculosis, Anthrax. Diarrhoea can suggest Salmonellosis, Colibacillosis etc. Nine major zoonotic diseases, or classes of diseases, are accorded priority status in India by Roadmap to Combat Zoonotic Diseases in India (RCZI). Rabies is put up at the top among all, followed by anthrax, brucellosis, leptospirosis, zoonotic tuberculosis, Japanese encephalitis, cysticercosis, salmonellosis and rickettsial infections. Remaining zoonoses included food-borne illnesses, emerging viruses, and plague while 13 zoonoses are identified as most important, by International Livestock Research Institute (ILRL) i. e zoonotic gastrointestinal disease; leptospirosis; cysticercosis; zoonotic tuberculosis; rabies; leishmaniasis; brucellosis; echinococcosis; toxoplasmosis; Q fever; zoonotic trypanosomosis, hepatitis E and anthrax. Heavy economic loss is caused to a varying degree due to different type of zoonotic diseases present in India. For example based on epidemiological data of active surveillance programme it is estimated that due to Brucellosis there is loss of US $ 58.5 million per year and FMD causes $ 5 billion revenue loss in India. Therefore present paper describes a brief review on public health importance, epidemiology and economic impact of these important zoonotic diseases (as mentioned by RCZI) in India in order to describe their importance and urgent need to take preventive measures effectively for their control.

Rabies is one of the most important zoonotic diseases in India. Rabies is endemic in India (Sudarshan, 2004). More than 99% of all human deaths from rabies occur in the developing world including India (WHO, 1998). The endemic nature of rabies in India can be attributed to the prevalence of the disease in dogs as well as other species of domestic animals. Different scientists, organizations stated different datas on toll death of humans caused by rabies in India. According to the national survey by the Association of the Prevention and Control of Rabies in India (2003), it estimated that in India a total of 18,500 human deaths occur as a result of rabies each year. Whereas the World Health Organization (WHO, 2002) estimated that rabies caused 30,000 human deaths per year in India, which accounted for approximately 60% of the estimated global total of rabies deaths. While Sudarshan (2004) stated that since 1985, India has reported an estimated 25 000 – 30 000 human deaths from rabies annually. On the contrary, the Central Bureau of Health Intelligence found an annual average of 249 deaths. At the same time the loss of livestock due to rabies is significant, there are few publications on estimates of the incidence of rabies in livestock (Knobel et al, 2005). Ghosh (2006) stated that the majority of people who die of rabies are people of poor or low-income socioeconomic status. Prevention of human rabies is possible through mass dog vaccination, promotion of responsible dog ownership and dog population control programmes with a partnership approach but in India this is a big challenge as it has a large population of dogs (around 25 million) and very low vaccination coverage. The government of India has still not made rabies a notifiable disease, so many deaths go unreported.

Anthrax is a disease of herbivorous animals caused by *Bacillus anthracis*, and humans incidentally acquire the disease by handling infected dead animals and their products (Thappa and Karthikeyan, 2001, 2002; Hanna, 1998; Morton and Arnold, 2003). In the states like Chattisgarh and Orissa Tribals or any given community, particularly if underprivileged, eat carcass of dead animals. Such people are vulnerable. According to a recent review of literature, there have been about 205 documented cases from India, the majority (109) of cutaneous anthrax (Lalitha, 2001). The characteristic clinical features of cutaneous anthrax are a painless ulcer with surrounding vesiculation along with massive edema and eschar formation (malignant pustule). Due to underreporting the actual incidence of anthrax in India is not known accurately mostly (Lalitha, 2001). Penicillin is the drug of choice for all forms of anthrax, beta-lactamase producing strains of B. anthracishave been reported (Bradaric and Punda-Polic, 1992; Lalitha and Thomas, 1997). In order to prevent anthrax there is need for proper legislation for meat handling as well as effective immunization of animals (Lalita, 1996).

Leptospirosis is an infectious disease caused by *Leptospira interrogans* complex which has over 20 sero groups and more than 200 serovars. Leptospirosis are excreted in the urine of the animals and they affect man when he comes into contact with urine of infected animals, directly or indirectly, when he is exposed to an environment contaminated by the urine of the infected animals such as soil and surface water following monsoon rains (Dutta and Christopher, 2005). Therefore the illness commonly occurs during the monsoon. Leptospirosis has been under reported and under diagnosed from India due to a lack of awareness of the disease and a lack of appropriate laboratory diagnostic facility in most parts of the country. The outbreaks of leptospirosis have been reported from coastal Gujarat, Maharashtra, Kerala, Tamil nadu, Andhra Pradesh, Karnataka and Andaman periodically. In the last decade, there has been a rapid rise in the incidence of leptospirosis in north India (Sethi et al, 2010). Since 1970, occupational exposure accounting for 30% – 50% of human cases and recreational activities are recognized as important causes of disease. Severe Leptospirosis can be diagnosed by the presence of fever, jaundice and renal

failure. This is the pattern commonly seen in Kerala, Tamil Nadu and Gujarat. Atypical pneumonia has been reported from Andamans(Shivakumar,2013).

The presence of brucellosis in India was first established early in the previous century and since then has been reported from almost all states(Renukaradhya et al, 2002). In India Brucellosis is caused by mainly *Brucella abortus* and *Brucella melitensis* and is readily transmissible to man as an occupational hazard(Kollannur et al,2007). Public health significance of Brucellosis is well known (Koshi and Myers, 1969). The prevalence of brucellosis in cattle farms has long been recognised (Anon, 1918), and several studies have confirmed widespread prevalence in different States in India. Due to Brucellosis in India there is loss of US $ 58.5 million per year. Losses are due to abortion in the affected animal population, loss of progeny, infertility and reduced milk production. In humans, brucellosis is causing physical incapacity, loss of man days of labour. In India, effective control of brucellosis is a national problem.

Tuberculosis (TB) is one of the most ancient diseases of mankind and has co-evolved with humans for many thousands of years or perhaps for several million years (Hirsh et al, 2004). The zoonotic aspects of tuberculosis in human beings revolves around isolation of *Mycobacterium bovis* from their sputum specimen. Also it has been observed that not only *M. bovis* cause TB in animals but *Mycobacterium tuberculosis* too causes diseases in animals. The infection can get transmitted from animals to human beings and vice versa(Challu,2007). Prevalence of Bovine TB in India varies from 1.6% to 16% in cattle and 3% to 25% in Buffaloes(Mullick, 1994). In India, increase of *M. bovis* infection in humans has manifested into a grave public health problem (Cosivi et al,1998; Cousins et al,1999, Kazwala et al,2001).

Salmonellosis is a type of zoonoses, diseases which are transmittable from animals to human beings under natural circumstances (British Association for the Advancement of Science, 1977). Salmonellosis is one of the commonest and most widely disseminated diseases transmitted via food (WHO, 2005). In India many investigators have described the isolation of *Salmonella* from different sources (Ganguli, 1958; Khera, 1962; Sharma & Singh,1967). Places in India bear the highest risk of contracting salmonellosis during prolonged stays and can climb from 1∶30,000 in endemic regions to 1∶3,000 in high endemicity regions (Mayer et al, 2010). Case fatality rate due to salmonellosis has been varying between 1.1% to 2.5% in last few years. Regarding prevention, no effective vaccination program has been developed. Therefore, control must be based on a strict hygiene program. Education of food handlers in aspects of preparation, refrigeration, and cooking of foods of animal origin as well as personal and environmental hygiene.

Cysticercosis caused by larval stage of the tapeworm *Taenia solium*, is a major public health problem, especially in the developing world including India. Studies using neuroimaging techniques suggest that the disease burden in India surpasses many other developing countries. The annual societal cost (agriculture and health) of porcine cysticercosis/taenosis is estimated at about US $ 150 million in India alone(WHO,2013). There are certain unique features of the disease in India. The solitary form of the disease (solitary cysticercus granuloma, SCG) is the commonest presentation of the disease. Anywhere between 26% and 50% of all Indian patients presenting with partial seizures are diagnosed with a SCG on the CT scan (Wadia et al, 1987; Misra et al, 1994). The disease is prevalent in virtually all states of the country although it varies significantly between different states (Rajshekhar and Chandy, 2000). There are few reports of patients with cysticercosis from Jammu and Kashmir, a predominantly Muslim state, and Kerala where educational levels and hygienic standards are probably the highest in the country. Systematic population-based studies are lacking in most parts of the India; hence it is difficult to estimate the disease burden in India.

Japanese Encephalitis (JE) was first recorded in Vellore and Puducherry in the mid 1950's and the first major outbreak occurred in 1973 in Bankura and Burdwan districts of West Bengal before spreading to other states. This disease has been reported from 26 states and UTs since 1978, only 15 states are reporting JE regularly. Most human cases reported from May to October, especially in northern India. The clinical manifestations of the disease are characterised with high-grade fever, convulsion, confusion, stiffness of neck and altered levels of consciousness from stupor to deep coma. The fatality rate varies between 10% –40% and those who survive do so with various degrees of neurological complications like paralysis and cognitive deficiencies. In India the total population at risk is estimated 160 million. In India alone, the JE have claimed nearly 1,000 lives so far in 2012. The most disturbing feature of JE has been the regular occurrence of outbreak in different parts of the country. Govt. of India has constituted a Task Force at National Level which is in operation and reviews the JE situations and its control strategies from time to time.

Rickettsial diseases are zoonoses caused by obligate intracellular bacteria grouped in the order Rickettsiales. According to RCZI important rickettsial diseases in India are Scrub Typhus; Epidemic Typhus; Endemic Typhus and Tick Typhus. For India, the reported numbers are an

underestimate due to lack of community based data and non-availability of confirmatory laboratory tests (Chugh, 2008).

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Survival of LA-MRSA in Dust of Pig and Poultry Barns

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Summary: Dust of pig and poultry barns can be a carrier of culturable livestock associated methicillin resistant *Staphylococcus aureus* (LA-MRSA). A re-entrainment into pig herds or poultry flocks from a contaminated environment seems to be possible. However, the survival of LA-MRSA in the animal environment and the transmission pathways are not fully understood. Therefore we tested the survival of culturable LA-MRSA in 20 dust samples of pig barns and in 9 dust samples of poultry barns. All dust samples were stored in sterile glass tubes with cork plugs at 4℃ in the dark. The age of analysed pig dust ranged between 18 and 34 months and the age of analyzed poultry dust varied from 10 to 34 months. The detection limit was 100 cfu LA-MRSA/g dust. LA-MRSA was detected again in two pig dust samples after 21 and 32 months and again in 4 poultry dust samples after 10, 14, 14 and 17 months. The detection rate (0.10) was lower in the aged pig dust samples than in the aged poultry dust samples (0.44), probably due to the in general older pig dust samples. *Spa* typing of isolates from the stored poultry dust samples showed the same types (t011 and t034) as found in the original samples. In general the results indicate a loss of culturability in the course of time. Nevertheless, the detected long-time survival of LA-MRSA in animal house dust suggests an increased risk for a re-entrainment into a new herd or a new flock. The results underline the importance of effective cleaning and disinfection measures to avoid a re-entrainment from indoor dust residues and from the outdoor environment.

Introduction

Livestock associated methicillin resistant *Staphylococcus aureus* (LA-MRSA) is a potential zoonotic pathogen widely spread in German pig holdings and can also be found in turkey and broiler flocks. Recent studies showed that MRSA can be found in environmental samples such as boot swabs, litter, dust and air samples of LA-MRSA positive pig herds and poultry flocks [1, 2]. Moreover a deposition of LA-MRSA on surfaces within the vicinity of pig and poultry barns was observed and it is most likely that these surface contaminations were caused by dust emissions from the investigated animal houses [2, 3]. Thus re-contamination of cleaned and disinfected animal houses may occur by re-entrainment from dusty surfaces by persons (e. g. farmers), or by animals like rodents, which come into contact with these surfaces and subsequently enter the animal house. However, to assess these transmission risks there is a need for studies in order to understand the survival of LA-MRSA in animal house dust. So far there is no information about the survival time of LA-MRSA in dust. Laboratory experiments with hospital acquired MRSA (HA-MRSA) strains showed that MRSA inoculated in hospital dust survived up to approx. 10 months [4]. The chosen concentrations seem to be high and may not reflect realistic concentrations of MRSA in dust samples. This study reports about the concentration and survival of LA-MRSA in dust samples originated from pig and poultry barns.

Material and methods

20 pooled dust samples from 11 pig farms (A to K) and nine pooled dust samples from four poultry farms (A to D) were sampled from 20 different pig herds and 9 different poultry flocks. Samples were taken and analysed quantitatively and qualitatively (by enrichment) within 24 h as described by Friese et al. [1]. The detection limits were 1000 cfu/g dust for the quantitative analysis and 100 cfu/g dust for the enrichment method. After the first analysis all dust samples were stored by a constant temperature (4 ± 1)℃ in a cooling chamber. The age of dust samples from pig herds were 18 to 34 months and the age of dust samples from poultry flocks were 10 to 34 months when the second analysis was carried out (Table 1). Isolates from poultry farms B, C and D were typed by *S. aureus* protein A gene (*spa*) sequence typing [5].

Results and discussion

LA-MRSA was detected in all fresh dust samples by enrichment. The concentrations varied between 6,000 and 1,290,000 cfu/g (median 59,500 cfu/g) in the

dust of pig barns and between 4,000 and 480,000 cfu/g (median 70,000 cfu/g) in the dust of poultry barns. These results are comparable to other studies [1, 2]. After storage LA-MRSA could have been recultivated from 6 out of 29 samples. The age of positive tested dust samples from poultry flocks was 10, 14, 14 an 17 months and the age of positive dust samples from pig herds was 21 and 32 months. From five of these samples the number of culturable LA-MRSA could have been quantified. A quantification of LA-MRSA seems to be more likely in high contaminated and less old dust samples rather than in old dust samples with lower concentration. However, data are not sufficient to underline this assumption statistically. Considering the detection limits the decline of culturable LA-MRSA was more than 100 fold in most cases. *Spa* typing resulted in the same types in the first and second analysis of dust from poultry flock number 3 (t034), 4 (t011), and 8 (t011). The detected *spa* types t011 and t034 typically belong to the LA-MRSA of the clonal complex 398 [6].

Table 1 Quantitative and qualitative detection of LA-MRSA in fresh and aged dust samples from pig and poultry farms

Sample No. (Farm)	Date of first analysis	cfu/g	Detection by enrichment	Date of second analysis	Age of dust in month	cfu/g	Detection by enrichment
1(pig A)	21/09/2009	101,000	+	23/07/2012	34	–	–
2(pig B)	28/09/2009	81,000	+	23/07/2012	34	–	–
3(pig C)	26/10/2009	16,000	+	23/07/2012	33	–	–
4(pig D)	09/11/2009	430,000	+	23/07/2012	32	–	–
5(pig E)	23/11/2009	9,000	+	23/07/2012	32	–	–
6(pig F)	07/12/2009	15,000	+	14/08/2012	32	–	–
7(pig G)	25/01/2010	55,000	+	14/08/2012	31	–	–
8(pig G)	25/01/2010	92,000	+	18/09/2012	32	–	+
9(pig H)	02/03/2010	140,000	+	14/08/2012	29	–	–
10(pig I)	15/03/2010	28,000	+	14/08/2012	29	–	–
11(pig J)	19/04/2010	64,000	+	14/08/2012	28	–	–
12(pig I)	07/09/2010	10,000	+	06/09/2012	24	–	–
13(pig H)	21/09/2010	120,000	+	06/09/2012	24	–	–
14(pig K)	10/11/2010	1,290,000	+	18/09/2012	21	39,000	+
15(pig K)	26/11/2010	270,000	+	20/08/2012	21	–	–
16(pig K)	14/12/2010	100,000	+	20/08/2012	20	–	–
17(pig I)	17/01/2011	6,000	+	18/09/2012	20	–	–
18(pig H)	24/01/2011	54,000	+	06/09/2012	20	–	–
19(pig H)	24/01/2011	21,000	+	06/09/2012	20	–	–
20(pig K)	21/02/2011	45,000	+	20/08/2012	18	–	–
1(poultry A)	19/10/2009	480,000	+	27/08/2012	34	–	–
2(poultry B)	14/03/2011	50,000	+	27/08/2012	17	–	–
3(poultry C)	22/03/2011	20,000	+	18/09/2012	17	10,000	+
4(poultry D)	11/07/2011	overgrown	+	18/09/2012	14	51,000	+
5(poultry D)	11/07/2011	overgrown	+	18/09/2012	14	60,000	+
6(poultry B)	13/07/2011	4,000	+	27/08/2012	13	–	–
7(poultry D)	24/10/2011	70,000	+	05/09/2012	11	–	–
8(poultry B)	07/11/2011	280,000	+	18/09/2012	10	37,000	+
9(poultry D)	14/11/2011	340,000	+	05/09/2012	10	–	–

In general the results showed a strong decrease of the culturability of LA-MRSA in stored dust of pig barns and poultry houses in the course of time. The reason therefore could be the damage of *S. aureus* over time by desiccation, by starvation, by competition between other microorganisms or by chemical residues (e. g. pharmaceuticals) within the dust. The impact of these factors is probably influenced by the temperature and may be less at low temperatures. Otherwise higher temperatures increase the metabolic activity of *S. aureus* and dust of animal houses may also act as an energy source providing that a sufficient water activity is given. That means, under certain circumstances even a reproduction of *S. aureus* is conceivable. This should be taken into account in future studies. Neglecting a possible affect on the survival due to higher or changing temperatures this study delivers comparable results and indicates a considerable survival time of LA-MRSA in stored dust of animal houses. This indicates a high tenacity of LA-MRSA, which could lead to an accumulation within and in the vicinity of MRSA positive pig or poultry barns. Especially the dissemination via the airborne route is difficult to control and may lead to a contamination of all surfaces within and in the vicinity of pig and poultry barns. Therefore implementing strin-

gent bio-security measures are necessary to avoid a re-entrainment of LA-MRSA into a new herd or into a new flock. To monitor the cleaning and disinfection measures, taking swab samples and boot swab samples from the treated surfaces are recommended. The latter samples can also be used to test the surface contamination in the vicinity of barns [3].

Conclusions

The study indicates a high tenacity of LA-MRSA in dust of pig and poultry barns. To avoid a re-entrainment from former LA-MRSA positive pig and poultry barns into new herds and flocks is a challenge for farmers. However, the efforts of farmers to prevent a dissemination of LA-MRSA can only be successful when breeders deliver LA-MRSA free animals.

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Histopathological Studies on Human Cutaneous Leishmaniasis

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Summary: Leishmania, a genus of intracellular protozoan parasites of macrophages, is the etiologic agent of cutaneous and visceral disease in man. In our study, localized infections with leishmania were studied by light microscopy in 16 patients at phases ranging from onset to resolution of the disease.

In early infections, epidermal changes could be detected as deep haemorrahgic ulcer characterized by focal massive necrosis of the epidermal layers. Spongiotic vesicles in the epidermis were prominent containing the amastigotes. The dermal changes appeared in the form of inflammatory infiltrate predominantly composed of macrophages, epithelioid cells, lymphocytes and mast cells. Macrophages laden with parasites were seen dissociating the striated muscle and the collagen bundles which showed degenerative and necrotic changes.

In late stages of the disease, multiple granulomas formed predominantly of macrophages containing promastigotes and amastigotes, giant cells, epithelioid cells and some mast cells were seen in the dermis. Some macrophages appeared vacuolated. The dermal vasculature showed congestion, swelling of the endothelial cells and fibrinoid necrosis of the wall. Some congested blood vessels demonstrated margination and diapedesis of inflammatory cells. The results were interpreted and discussed.

Introduction

Leishmaniases are diseases caused by protozoa of the genera Leishmania. In the New World, the aetiologic agents of cutaneous and/or mucocutaneous leishmaniasis are a number of Leishmania species [7]. Extreme cases of cutaneous infection are characterized by widespread dissemination of highly parasitized macrophages and unresponsiveness (diffuse cutaneous leishmaniasis, DCL) or by extensive destruction of nasopharyngeal tissues and hyperresponsiveness to scanty organisms (mucocutaneous leishmaniasis, MCL) [9]. More commonly, cutaneous leishmaniasis (CL) appears as a localized lesion, usually self-healing or responding well to treatment. Recovery is often followed by strong specific immunity and delayed hypersensitivity [5]. The course of leishmaniasis depends primarily on macrophage-parasite interaction [13,17,18]. Ultrastructural studies have documented the relationship of the parasite to the inflammatory cellular response [3,11].

The aim of this paper is to describe the histopathological changes of the observed cutaneous lesions both in early and late infections. In addition, the types of the macrophagal- histocytic cellular reaction and their role in the observed lesions is evaluated.

Material and methods

Patients: Sixteen patients with cutaneous leishmaniasis, being of differentages and sex, were diagnosed on the basis of clinical features and history. The clinical data of early and late infection were described in a previous article (data not shown). None of these patients received treatment before biopsies were taken after written consensus.

Processing of biopsy material: Four-millimeter punch biopsies were taken under local anaesthesia from the edge of ulcers or nodules and fixed in 10% neutral-buffered formalin. Tissues were routinely processed and embedded in paraffin. Five-micron sections were cut and stained with haematoxylin-eosin for histopathological examination by light microscopy. Giemsa and toulidine blue stain were used whenever needed.

Results and discussion

Epidermal changes: Light microscopic examination revealed prominent haemorrhagic ulceration associated with coagulation necrosis of the epidermal layers in some examined cases (Fig. 1A). Similar lesion were described by [4,10]. This may be of ischemic origin and have been considered one histologic correlate of DTH [12]. Along with these changes, intraepidermal spongiotic vesicles were observed in the epidermis. These vesicles characterized by lytic necrosis and mononuclear cellular infiltrate that contained the protozoal organism (Fig. 1B). Similar findings were described by [2,9]. Free amastigotes were also found between the epidermal layers and in the vesicle. These organisms were ovoid with rounded nucleus surrounded by a clear halo. Neither acanthosis nor hyperplasia of the epidermis was observed.

Dermal changes: In all examined cases, the small

arteries within and around the inflammation revealed swelling of endothelial cells, necrosis of the smooth muscles cells as well as subintimal fibrinoid changes. The presence of congested blood vessels with marginalization and diapedesis of inflammatory cells were a prominent finding (Fig. 1C) [6].

Diffuse lymphohistiocytic inflammatory reaction involving the dermis and subcutis was a common finding in the examined cases. The diffuse dermal infiltrate composed of macrophages, multinucleate giant cells, plasma cells, mast cells and rare eosinophils (Fig. 1D). When parasites are numerous and viable, they result in activation of macrophages and intracellular killing of their parasitic load [8]. Killing may be due to the generation of reactive oxygen species, superoxide and hydrogen peroxide or to effector lymphokine mediation [14]. The presence of mast cells in the dermis during infection caused by leishmania is related to their direct participation in the initial immune response through the production of several inflammatory mediators [16]. Large amount of vacuolated macrophages were seen containing many amastigotes while free proamastigotes and amastigotes were detected in the extracellular matrix (Fig. 1D). Degenerative and necrotic changes were

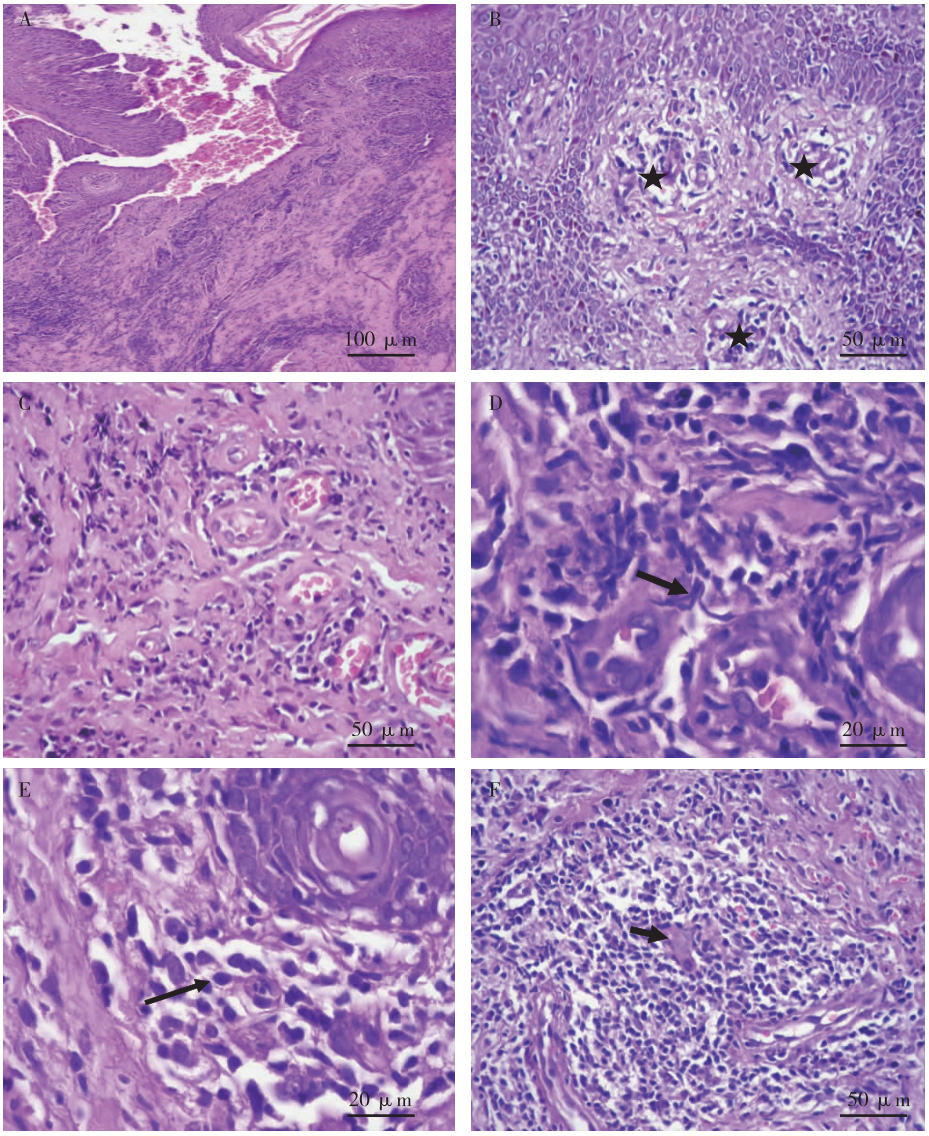

Fig. 1 (A) Showing deep epidermal haemorrhagic ulcer, H&E. Bar = 100 μm. (B) Showing spongiotic vesicle and parasitized macrophages (stars), H&E. Bar = 50 μm. (C) Showing congestion, degeneration of dermal vessels, marginalization and diapedesis of the inflammatory cells (arrow), H&E. Bar = 50 μm. (D) Showing diffuse dermal inflammatory reaction and free proamastigotes and amastigotes (arrow), H&E. Bar = 20 μm. (E) Showing degenerative and necrotic collagen and phagocytosed amastigotes (arrow), H&E. Bar = 20 μm. (F) Showing dermal granulomatous reaction and free amastigotes (arrow), H&E. Bar = 50 μm.

observed in the dermal collagen accompanied by histiocyatic macrophagal reaction that demonstrated the presence of amastigotes in these macrophages (Fig. 1E). Collagen changes may be due to ischemia produced by the vasculitis.

A granulomatous pattern of dermal lesions was demonstrated in the deep dermis of some cases. These granulomas composed of macrophages, epithelioid cells, few giant multinucleated cells, lymphocytes and mast cells (Fig. 1F). Few leishmania amastigotes were identified in the cytoplasm of macrophages. The granulomas were surrounded by delicate fibroblastic capsule. Granulomatous reaction associated with moderate, mixed inflammatory infiltrate with few leishmania amastigotes in the macrophages to an extensive inflammation composed of vacuolated macrophages containing large number of parasites were described by many authors [1,15]. The same authors added that multinucleated giant cells found in granuloma are related to the effect of interferon y and interferon y is known to be effective in activating macrophages to kill leishmanias intracellularly.

Conclusions

From our findings, it could be concluded that cutaneous leishmaniasis exhibited both chronic diffuse inflammatory reaction as well as nodular (granulomatous) pattern. Skin ulceration and epidermal coagulation necrosis was not accompanying all the examined cases. Vascular lesions played an important role in the pathogenesis of the developed lesions. The lymphohistocytic macrophagal reaction seemed crucial in defending the host against the protozoan parasite through different mechanisms.

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Current Scenario of Leptospirosis in India

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Abstract: Since 1994 Leptospirosis has emerged as a major public health problem in west coast of India. Studies in the endemic states revealed that males suffer more frequently from Leptospirosis than females within an age group of 20 – 30 years because of their occupation. In the urban areas Leptospirosis usually coincides with natural calamity like floods. In the recent past there is change in the clinical presentation of Leptospirosis. There is multi organ involvement with predominant sign of Acute Respiratory Disease Syndrome in many of the cases.

Keeping in view the increasing morbidity and mortality, a pilot project was undertaken in the endemic states. The strategy implemented for control of Leptospirosis were strengthening of Diagnostic facility, strengthening of patient management facilities, strengthening of IEC activities and training of professionals both doctors and laboratory technicians at the periphery health services. Chemoprophlayaxis and rodent control was also used extensively in many parts of Leptospirosis endemic areas with varying success. The implementation of the strategies helped to understand the changing pattern of the disease in human beings and also about the patient management of cases due to Leptospirosis.

Need of Intersectoral Coordination for Rabies Control in India

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Abstract: Canine rabies is a global public health concern and is responsible for most human rabies deaths. Rabies though a fatal disease, yet it carries the advantage that it is entirely a preventable disease. In a developing country like India all can't afford the cost of the post exposure prophylaxis. Ethical and cost effective antirabies vaccination via Intra dermal route has being introduced in some cites of India with success. Reducing cost of vaccination for human is a positive sign but still rabies elimation would be a fry cry if proper animal control strategy is not implemented.

Preventing human deaths through canine rabies elimination is feasible. Stakeholders, NGOs, animal welfare organizations should work on the strategy for the dog census, vaccination and animal birth control. Intersectoral coordination and awareness programme should be developed for both human and animal sectors for a successful rabies control programme. Here, we demonstrate that the responsibility of managing rabies in India should have an integrated intersectoral approach.

Modeling the Transmission Dynamics of Brucellosis in Inner Mongolia Autonomous Region, China

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Abstract: Brucellosis is one of the major zoonotic diseases in China, especially in Inner Mongolia which has the largest number of human brucellosis cases in Mainland China. The most important reason is that there is a very large number of sheep and goats, and at least 90% of the human brucellosis cases are caused by sheep and goats. So this paper combines the reported data and characteristics of the brucellosis infection in Inner Mongolia Autonomous Region to propose a mathematical model which includes sheep and goat population, human population and brucella in the environment. Based on the group classification, we consider sheep and goat-to-sheep and goat, sheep and goat-to-human, brucella in the environment-to-sheep and goat and human transmission of brucellosis. We first determine the basic reproduction number R0 and discuss the global stability of the disease-free and endemic equilibrium. Secondly, we carry out numerical simulations and sensitivity analysis of the basic reproduction number in terms of some parameters, the results show that brucellosis cannot be controlled even though disinfection rate and vaccination rate to adult sheep and goats are 100%. By investigating and comparing the effect of vaccination, disinfection and eliminating strategies, we find that both young and adult sheep and goat vaccination and disinfection are appropriate strategies to control brucellosis in Inner Mongolia.

Long-term Monitoring of ESBL-producing Enterobacteriaceae in Poultry and Pig Farms and in Their Vicinity

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Abstract: Antibiotic resistance is a major problem in both human and veterinary medicine. Especially MRSA and recently ESBL are identified as emerging problems for public health.

To investigate epidemiology, intra-herd kinetics as well as environmental impact in this on-going project, long-term investigations concerning the occurrence of ESBL-/AmpC-producing Enterobacteriaceae are carried out inside and in the vicinity of seven pig and broiler fattening farms, each. Therefore, one barn of each farm is investigated three times within one fattening period (at the beginning, in the middle and in the end). The results show a high occurrence of ESBL-/AmpC-producing Enterobacteriaceae, especially *E. coli*, inside the barns and also findings in the surrounding of the farms. Even a large part (51%) of the one-day-old chicken were tested positive for these resistant bacteria which leads to the question of the origin of these resistances and of vertical transfer, respectively. Concerning pig farms the detection level of ESBL-/AmpC-producing resistant *E. coli* was also very high. The investigated pig farms differ in their detection frequency of the ESBL-/AmpC-producing *E. coli*, some farms had a continuous low frequency in the animal samples others a very high level over all sampling times. Sampled broiler farms showed all high levels with a mean of 76% ESBL prevalence (min 25%, max 100%) at the end of the fattening period. Also the animals direct environment inside the pig and broiler barns was tested positive for ESBL-/AmpC-producing Enterobacteriaceae which could be one important transmission way of the bacteria within a flock. The frequent findings of these resistant microorganisms in dust samples originating from broiler farms in contrast to them from pig farms is striking. In air samples, however, ESBL-/AmpC-producing resistant *E. coli* were found rarely. In parallel, surfaces of the ground around the barns as well as exhaust air samples were taken. ESBL-/AmpC-producing resistant *E. coli* were infrequently detected on the ground in different distances from the barn, furthest in 500 m. Only very few samples of exhaust air were tested positive. In principle, emissions of ESBL-/AmpC-producing Enterobacteriaceae via the spread of dust but more probably via the faecal way (fertilization of fields) seem likely.

The Abnormal Distribution of Prion Proteins in Sheep after Infection with a Mouse-adapted Strain of Scrapie

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Abstract: Here we investigate the stability of mouse-adapted scrapie strains, RML and 22L, across the species barrier. Eight Small-tail Han lambs with various prion protein genotypes (3 ARQ/ARQ, 4 ARQ/ARH and 1 ARQ/ARK) were equally divided into two groups of 4 sheep. These groups were inoculated intracerebrally with a brain suspension from RML-infected or 22L-infected mice respectively. The mean survival period was 32. 7 months (RML-inoculated group) and 26 months (22L-inoculated group). During this time only 1 sheep in each group developed pruritus. Abnormal prion protein deposits in the central nervous system, the retina and the terminal ileum of the infected sheep were detected. The PrPSc staining in the cerebrum of the RML group was more severe than that in the 22L group. In addition, the distribution of PrPSc was markedly different in the cerebrum between the 22L and RML groups. Interestingly, typical pineal body vacuoles were found in one quarter of both the RML-inoculated sheep and the 22L-inoculated sheep. Abnormal prion proteins were present in half of the pineal bodies of the RML- inoculated sheep. This study also shows that the distribution of abnormal prion protein in lymphoid tissues is distinct to that seen in classical scrapie, but partially similar to atypical scrapie. These findings demonstrate that the mouse-adapted scrapie strains RML and 22L can overcome the species barrier thus inducing experimental scrapie in sheep.

Immunohistohemical and Pathological Studies of Rabies in Brain of Domestic Animals in Pakistan

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Abstract: Rabies is a horrifying neglected infectious disease and a severe underestimated global health issue. Approximately, 70,000 people died of rabies every year primarily in Asia and Africa, where dog rabies is enzootic. The aim of present study was to investigate the pathological and immunohistological detection of rabies in brains of domestic animals of district Faisalabad, Pakistan. A total of 192 brain samples, consisting of six samples of each species (Sheep, goat, cow and buffalo) were collected from eight towns including (Madina, Jaranwala, Iqbal, Layallpur, Sumandri, Tandlianwala, Chak jhumra, and Jinnah). Grossly brains of the affected animals were severely congested and edematous microscopically, the cerebrum and cerebellum showed characteristic lesions including degenerative and necrotic changes in the glial cells and neurons, rod shape neurons, perivascular cuffing with characteristic Babe's nodule and gliosis. The purkinje cells were severely affected with intracytoplasmic virus inclusions throughout the cerebellum. The neuronal layers of the hippocampus were severely infected with the rabies virus and the viral particles were accumulated in the cytoplasm of their perikaryons. Out of total samples only 2 (2.08%) brain tissues which were of goat and cow belonging to the Tandlianwala and Jaranwala town were diagnosed positive for rabies virus and rest of the samples 190 (97.92%) were negative for the rabies virus. Furthermore, future study must be carried out on the sequencing, genomic characterization of the nucleoprotein of the rabies virus which would definitely be very helpful to study the circulating viral isolates and to lay down solid foundation for preventing of rabies in Pakistan.

Campylobacter jejuni and *Campylobacter coli* in Organic Turkey Flocks

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Abstract: Consumers and farmers have been increasingly interested in organic food products. They assume that organic products are safer and healthier. However, there are also aspects of organic animal husbandry, like access to an outdoor run, which can result in increased risks of food safety problems. Therefore, we perform this study to determine the prevalence and *flaA* types within flock and possible source of infection. In each 5 organic turkey flocks (4 – 8 weeks old), samples were collected from cloacal swabs (n = 30 birds), 10 pooled water samples (main water tank n = 5 and drinkers n = 5) and darkling beetles. All 5 flocks were *Campylobacter* positive from cloacal swabs. *Campylobacter* was isolated from 147 of the 150 cloacal swabs samples (98%), 3 of the 15 drinkers (20%) samples (flocks 1and 2) and internal contents of litter beetles in flock 2. No *Campylobacter* was isolated from main water tank. Both *C. jejuni* and *C. coli* were recovered from the cloacal swabs with prevalence among flocks ranged from 27% to 63% for *C. jejuni* and 27% to 73% for *C. coli* as well as from drinkers. Only *C. coli* was isolated from litter beetles. *Campylobacter* isolates recovered were analyzed for *flaA* typing. Different genotypes were obtained, which varied from 2 to 4 genotypes for *C. jejuni* and 1 to 4 genotypes for *C. coli*. This study revealed that organic turkey flocks are often contaminated with *Campylobacter* with high prevalence at early age. Also, the contaminated water and darkling beetles can play a role in the entry of *Campylobacter* into turkey flocks. A more through understanding of the relationship between beetle infestation, water contamination and the *Campylobacter* status of organic turkey flocks should enable progress in further development of biosecurity control measures.

Feed and Water Quality

A Longitudinal Study to Assess the Hygienic Quality of Drinking Water for Animals in Germany

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Summary: The provision of safe drinking water is essential to the health, welfare, and efficient production of animals. However, the role of water as an essential nutrient for the animals is often underestimated. The quality of drinking water is defined by microbiological, chemical and physical factors. Water can serve as a reservoir for many different microorganisms and chemical compounds contributing to eventually diseases. Presently there are no specific legal regulations which define the quality of the drinking water for animals in Germany. However, guidelines exist which define quality limits and help to secure the drinking water supply for animals.

Although risk factors associated with hygienically relevant microorganisms or chemical compounds in drinking water for animals are known from the literature, information on the drinking water quality for animals are limited. In order to gain an overview of the chemical and microbial contamination of drinking water used for livestock, data were generated from March 2004 to October 2012. In total, 273 samples were chemically analyzed and 135 samples were microbiologically analyzed. This dataset exceeded the chemical guideline values most frequently. Iron values varied from 0.03 mg/l to 101.0 mg/l, nitrite ranged from 0.01 mg/l to 142.0 mg/l, and sulfate was 1.94 mg/l to 1110.0 mg/l. The number of samples exceeding chemical guideline values (88.4%) was higher than the number of samples exceeding microbiological guideline values (31.9%).

The present study highlights the possible risk for chemical compounds in drinking water for animals and indicates the need for hygienic measures to prevent the spread of microorganism as well as chemical compounds via water.

Introduction

Provision of good drinking water quality is important to improve health, welfare, and efficient production of animals. Considering that relative to other nutrients water is consumed in larger quantities, drinking water may serve as potential health risk for animals. Therefore, limited water availability and poor quality drinking water may depress animal production including milk yield or growth rate significantly.

Although risk factors associated with hygienically relevant microorganisms or chemical compounds in drinking water for animals are known from the literature [1 – 4], information on the drinking water quality for animals are limited. Our investigation was performed to gain an overview of drinking water quality used for livestock. In a longitudinal study chemical and microbial contaminations were analyzed. The aim of the present study was to contribute to a more detailed understanding of the whole extent of drinking water quality for animals. It highlights the possible role of poor water quality as a source of disease transmission and indicates the need for further monitoring and hygienic measures.

Material and methods

Drinking water samples were collected from farms located in the north-western part of Lower Saxony, a region with the highest livestock density in Germany. Data were generated from March 2004 to October 2012. In total, 273 samples were chemically analyzed and 135 samples were microbiologically analyzed. Standard methods were used to analyze chemical and microbiological water quality parameters. Microbial organisms were measured as colony forming units (CFU) using a plate counting method. Results were compared with the prescribed Federal Ministry of Food, Agriculture and Consumer Protection guidelines.

Results and discussion

During the longitudinal study, analysis of water samples gained an overview of chemical and microbial contamination of the current drinking water quality of animals (Table 1).

Table 1 Water samples chemically (n = 273) and microbiologically (n = 135) analyzed

Parameter	Mean[a]	Max[a]	Min[a]	Exceeding D[b] (%)	Exceeding N[c] (%)
pH	6.36 ± 0,96	8.40	3.31	18 [6.6]	87 [31.9]
Hardness	11.68 ± 10.17	90.00	0.40	58 [21.3]	58 [21.3]
Ammonium	1.61 ± 4.15	50.10	0.04	33 [12.1]	64 [23.4]
Chloride	51.41 ± 82.15	1087.00	1.27	1 [0.4]	1 [0.4]
Iron	4.67 ± 10.68	101.00	0.03	95 [34.8]	159 [58.2]
Nitrate	29.53 ± 43.93	224.00	0.01	1 [0.4]	84 [30.8]
Nitrite	0.87 ± 9.33	142.00	0.01	2 [0.7]	40 [14.7]
Sulfate	69.73 ± 103.76	1110.00	1.94	4 [1.5]	45 [16.5]
CFU/ml 20℃	1,686 ± 3,533	20,992	0	3 [2.2]	23 [17.0]
CFU/ml 37℃	1,714 ± 3,365	14,336	0	23 [17.0]	44 [32.6]

[a] mg/l; [b] In accordance with the German guidelines; [c] In accordance with the Dutch guidelines.

The number of samples exceeding chemical guideline values (88.4%) was higher than the number of samples exceeding microbiological guideline values (31.9%). In total, 80 water samples were analyzed for chemical and microbiological contaminations simultaneously. There was no statistically significant correlation between water samples exceeding chemical and microbial guideline values, indicating the association that chemical components does not necessarily increase the risk of microbial contamination.

According to analyses of 273 drinking water samples from livestock production farms located in north-west Germany, iron seems to be the most frequent water quality concern regarding chemical contamination in drinking water for animals. Iron values varied from 0.03 – 101 mg/l with 35% (95/273) exceeding guideline values (< 3 mg/l) defined by the German Federal Ministry. Considering that concentrations of greater than 0.2 mg/l are considered to be a risk for animal's health by the Dutch Federal Ministry, even 58% (159/273) of the tested water samples would not be suitable for farm animals. It is known that higher amounts of iron in drinking water may influence the acceptability and thus reduce the rate of water intake [1]. Although few studies were published, reliable research on the influence of iron in drinking water on different farm animal's behavior and performance is limited.

Nitrate and nitrite occurring naturally in water are oxidized forms of nitrogen. Nitrate ranged from 224 – 0.01 mg/l and nitrite ranged from 142 – 0.01 mg/l. Previous studies demonstrated that an increased concentration of nitrate in drinking water does not have any injurious effect on the health of weaned piglets [2]. However, nitrite in drinking water significantly reduced average daily gain and feed consumption [3]. According to the present study increased nitrite values in drinking water with 1% (2/273) is rather rare.

Sulfate guideline values differ between countries. With 1.94 – 1110 mg/l sulfate in the tested water samples 1.5% (4/273) and 16.5% (45/273) exceed preferred values for Germany and the Netherland, respectively. Thus, standardized values for European countries are recommended.

Water pH (1 – 14) describing the acidity or alkalinity of a source, is not supposed to have a major influence on water acceptability. Generally drinking water with a pH 6 – 9 is considered to be acceptable for livestock. Slightly different suitable pH values between 5 and 8 were suggested by the Federal of Netherlands. Accessibility as well as impact on animal health correlating with pH value of drinking water may vary among species and production mode. Our results demonstrated that analyzed water samples exceeded suitable values most frequently (DE 7% / NL 32%). Although the specific pH's effect on water intake or animal health is unidentified, high or low pH values may significantly impair the efficiency of certain water treatment systems such as chlorine with an optimal pH of 5 – 6.

Water hardness indicates the sum of calcium and magnesium. In the present study, more than 21% (58/273) of the tested water samples had a hardness level greater than 15°dH. There seems no reliable study investigating water hardness and its impact on animal health. However, it has to be considered that hard water may cause calcification leading to biofilms.

Microbiologically analyzed water samples had up to 20,992 CFU/ml incubated at 20℃ and up to 14,336 CFU/ml incubated at 37℃. In total 32.5% and 57.5% of the tested water samples exceeded German and Dutch guideline values, respectively. Currently the suggested values for total bacteria at 20℃ are < 10,000 CFU/ml in Germany and < 1,000 CFU/ml in the Netherlands. Considering that animals drink a considerable amount of water, drinking water as a source of disease transmission is often underestimated. From the literature it is known that biofilms offer an optimal habitat for pathogenic microorganisms such as bacteria, viruses and parasitic protozoa [4]. Hygienically relevant microorganisms can settle to preexisting biofilms and may represent a source

of water contamination resulting in a potential health risk for animals. Thus, it seems necessary to clean drinking water installation system periodically.

Conclusions

Water availability and quality are essential for animal health, especially regarding highly producing livestock. Contaminants in drinking water increases with water intake, therefore, it is essential to know that the daily intake varies with species, production mode or climate. This study highlights the possible risk for chemical compounds in drinking water for animals and indicates the need for hygienic measures to prevent the spread of microorganism via water. Regular monitoring of drinking water quality and the implementation of hygienic prevention measures are highly recommended. Further investigations are required in order to determine the potential risk regarding poor drinking water quality. This includes surveys on water quality and its effects on water intake as well as animal health.

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Study on Decontamination of Silage from *Paulownia elongata*

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Summary: Studies for the presence of pathogenic microflora and for the time of decontamination of ensiled and fresh leaf of *Paulownia elongata* were carried out. For this purpose changes in the quantities of microorganisms contained in the material, as well as of pathogenic test strains of *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*, differing in resistance to gentamicin and tetracycline antibiotics were tracked. Test bacteria were introduced into silage and foliage in quantities of 10^5 CFU/g of the total content. It was found that the microflora of leaves of *P. elongata* is presented principally by cocci and bacilli. *E. coli*, *Salmonella enterica* and *Clostridium perfringens* were not isolated. In silage were found mainly lactobacilli. The imported *Pseudomonas aeruginosa* survived in the silage 3 days and the test strains from other species – 10 days. The test bacteria remained longer in the fresh leaves – within 3 to 4 weeks. The ensilaging provides fast and safe decontamination of leaf of *P. elongata*. Use of such silage for feeding of animals is safe from epidemiological perspective.

Key words: *Paulownia elongata*, silage, microflora, test bacteria, decontamination

Introduction

Paulownia elongata is rapidly developing plant suitable for timber, which in recent years began to grow in Bulgaria. It provides a large amount of foliage. The leaves can be used to feed herbivorous animals (cows, sheep, goats, etc.). Their nutritional value is similar to that of alfalfa. They contain 20% protein in green and about 12% after the autumn fall of the leaves. The leaves are rich in valuable micronutrients and their digestibility is 60% (Angelov, 2010). Mueller et al. (2001) reported very good results in feeding the goats in the U. S. with such feed. The leaves are very tasty and have enough quality herbage for feeding goats. According Hongfu et al. (1995) nutrition of the fallen leaves surpasses that of straw from rice or wheat. In China, they are widely used as a cheap source of feed to pigs and ruminants. The feed by *P. elongata* is of high quality and favorable levels of energy and protein (Boying, 1995). Tendency to use it there is in our country too.

In some cases the fallen leaves can be carriers of different microorganisms, including pathogenic for animals, coming from the soil and fertilizer, and spread by rodents. Therefore this work focuses on tracking the survival of pathogenic test microorganisms imported into silage by *P. elongata*, in order to assess the potential for decontamination and epidemiological safety in its provision for animal feed.

Material and methods

Feed: Fresh and silage leaf of *Paulownia elongata* were examined.

Microorganisms: Pure cultures of three pathogenic bacterial strains were used: *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*. They were isolated from animals with chronic infections and were selected by the demonstrated resistance *in vitro* to gentamicin and tetracycline antibiotics (Tetracycline, Doxycycline and Oxytetracycline). A further cultivation of these strains was done on nutrient media with antibiotics from these groups in order to isolate and use in research branches, the most thriving in the presence of high concentrations of these antibiotics.

Nutrient media (from Antisel-Sharlau Chemie S. A., Spain): For the isolation and cultivation of test bacteria were used selective media containing both doxycycline at a concentration of 50 μg/ml and gentamicin – 16 μg/ml. Eosin Methylene Blue agar for *P. vulgaris*, Cetrimide agar for *P. aeruginosa* and Chapman Stone agar for *S. epidermidis* were selected. The total number of microorganisms in the studied materials was reported on Mueller Hinton agar without antibiotics. Contents and quantities of *Clostridium perfringens* on selective agar (Merck-Bio Lab, Bulgaria), of the coliforms and of *Salmonella enterica* on *Salmonella-Shigella* agar and Eosin Methylene Blue agar were also tracked.

Quantification of microorganisms was performed by a traditional way in increasing tenfold serial dilutions of the tested materials in a sterile saline solution. Cultures of them have been made on the media with and without antibiotics, three for each medium and dilution. After incubation at 37℃ for 24 – 48 h under aerobic and anaerobic conditions (using anaerob pack with palladium

catalyst – H_2 + CO_2 – Bul Bio NCIPD – Sofia) the average number of developed colonies was counted and the amount of the colony forming units (CFU) per 1 g of feed was calculated.

Microscopic studies of some microorganisms were carried out under immersion at a magnification of ×1200 after staining by different classical methods (Gram, Klett for capsules and Moeller for spores) of materials from different cultures on nutrient media.

Experimental set-up: Tested samples of fresh and ensiled leaves of *P. elongata* were placed of 200 g each in glass containers and stored at about 22℃. After a preliminary determination of the number of bacteria from groups *E. coli* and other gram-negative aerobic and facultative anaerobic bacteria (coliforms), *Pseudomonas* spp., *Staphylococcus* spp. and the total number of microorganisms in fresh and ensiled leaves in each of the materials were imported indicator microbial strains, each in quantity of 10^5 CFU/g of total content of the material. Samples for quantification of microorganisms are taken weekly from the first to the sixth week after the introduction of the test bacteria.

Statistical analysis of the results was carried out using the classical method of Student-Fisher.

Results and discussion

Studies on the microflora of leaves and silage of *P. elongata* did not show presence of pathogenic microorganisms. The data are shown in Table 1 and Fig. 1. The microflora of the leaves was consisted mostly of bacilli. Some cocci were established too, dominated by non-pathogenic tetra cocci. Unless them in silage were identified and oval fungi.

Table 1 Microflora of leaves and silage of *Paulownia elongata*

Groups of microorganisms (CFU/g)	Material	
	Leaves	Silage
Total number	6.2×10^{8} * ± 0.5 **	8.3×10^{6} ± 0.2
E. coli	–	–
Salmonella enterica	–	–
Pseudomonas spp.	–	–
Staphylococcus spp.	1.2×10^{3} ± 0.2	5.7×10^{4} ± 0.7
Enterococcus spp.	–	–
Clostridium perfringens	–	–

* Average. ** Standard deviation.

pH values of silage and leaves were 4 and 6.5 respectively. As seen from the data in the table, samples of silage and leaves of *Paulownia elongata* did not contained *E. coli*, as well as *Salmonella enterica* and *Clostridium perfringens*. This shows that their provision as feed is safe for animals from epidemiological viewpoint and do not present a risk of transmission of pathogens. Since the experimental leaves were picked directly from the trees, they were not exposed to contamination with soil or manure, which could occur during routine methods of collection and storage. Probably for this reason, *E. coli*, *Enterococcus* spp. or *C. perfringens* were not isolated from them.

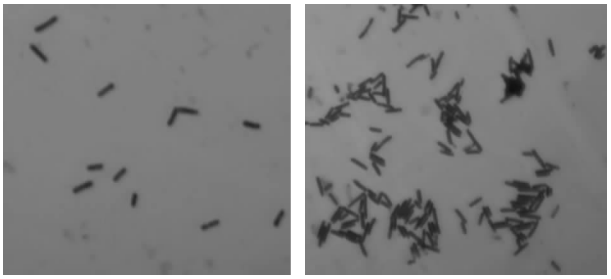

Fig. 1 Microflora of leaves (a) and silage (b) of *Paulownia elongata* (Gram staining, ×1200)

Test results of the investigations of silage by *P. elongata* for decontamination in terms of imported test strains of pathogens are presented in Fig. 2. As seen from the figure, the indicator *Pseudomonas aeruginosa* survived in silage only three days, and the test strains from other species—almost 10 days. This is an indication of the safety of such silage for animal consumption. Even if pathogenic microorganisms were fall into this feed from the environment or from contamination by rodents, inactivation could occur in a short time—less than 10 days.

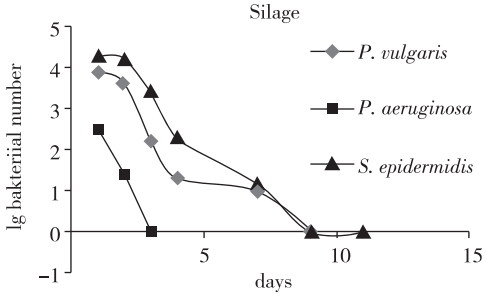

Fig. 2 Dynamics of test microorganisms in silage of *Paulownia elongata*

In the non ensiled leaves test bacteria remained for a longer time—three to four weeks. This can be seen from Fig. 3, which presents data from studies of leaves of *P. elongata* for decontamination after submitting the pathogenic test strains.

The results obtained from these tests showed that the leaves and silage of *P. elongata* normally do not contain pathogenic microorganisms and their provision for food of animals do not poses a risk of food-borne infections. Apparently on the leaves there are not favorable conditions

for growth of pathogenic bacteria such as *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*, as after their introduction in the leaves collected from this kind of wood their quantities gradually reduced and after one month they are no longer set in the material.

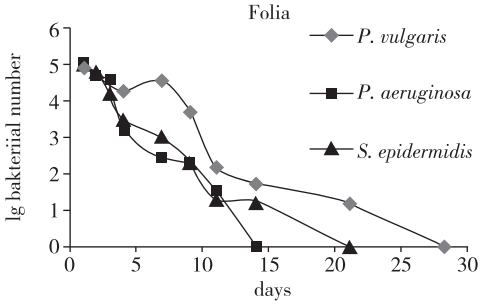

Fig. 3 Dynamics of test microorganisms in the leaves of *Paulownia elongata*

Decontamination of the silage was done quickly. Even after the introduction of pathogenic bacteria in it, they died in less than two weeks. Apparently this was due to the low pH of the silage and to the competitive relationships with other organisms. These data suggest that ensilage provides fast and reliable decontamination of foliage of *P. elongata*. Undoubtedly, the use of such silage for feeding is safe from epidemiological perspective.

Conclusions

The leaves and silage of *Paulownia elongata* normally do not contain pathogenic microflora.

Ensilage provides fast and safe decontamination of leaves of *Paulownia elongata*. The imported in silage pathogenic test bacteria *Pseudomonas aeruginosa* die for 3 days, and *Proteus vulgaris* and *Staphylococcus epidermidis* within 10 days.

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The Use of Sweet Whey for Weaning Pigs

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Summary: Sweet whey is a product that could be used in different farm systems for its high nutritional content, food quality, his appetite and palatability. Tests were carried out with weaning piglets, while measuring their growth and development, to replace partial (25% and 50%) and completely (100%) water supply by sweet whey. These biological tests were performed in a swine farm in the town of Puente Grande, in Jalisco State, Mexico. The experiments: 64 piglets were chosen randomly, although maintaining uniformity in weight, sex and age. They were distributed into four pens of 16 piglets each. Two groups of 8 piglets, were chosen for a different treatment. Each set was provided with the hardware required for the supply of sweet whey. The interpretation was done under completely randomized design. Obvious differences in the middle of treatments, were checked by using the Tuckey test. The variables that were measured in this experimental process were: weight gain, food consumption, feed conversion and consumption of sweet whey; the latter in order to be able to check the results of the repetitions and to get to know the proportion (%) of more convenient sweet whey. We recommend using sweet whey in proportion of 25% and 50% of the total liquid supply in the diet.

Key words: sweet whey, weaning, weight gain, feed conversion, piglet

Introduction

The dairy whey is a liquid sub product of the dairy industrialization, slightly acidic and with a yellowish green color, residual of the milk coagulation by application of rennet or by reducing its pH.

The characteristics of the whey vary according to the milk which is employed and the method of coagulation.

Are distinguished two main types of whey:

1. Sweet whey.
2. Acidified whey.

The sweet whey comes from coagulated milk whit rennet (coagulating enzyme of animal origin called renin).

The acidified whey comes from the manufacture of soft cheeses, or by adding dilute organic or inorganic acids. This whey presents a reduction of the lactose caused by the lactic fermentation, and therefore is considered of lower quality than the sweet whey.

The proteins of the whey (lactalbumin and globulin) are of excellent quality for its functional properties since the amount of essential amino acids is higher than the proteins of the egg. It also contains casein proteins, very rich in lysine and tryptophan amino acid with sulfur (methionine and cystine).

Treatment description:

Treatment 1. (Witness.) Balanced feed was provided produced on the farm itself and free access to water.

Treatment 2. (25%.) Balanced feed was provided, produced on the farm itself and 25% of the sweet whey, in a proportion of 0.250 L, per day, by piglet and free access to water.

Treatment 3. (50%.) Balanced feed was provided, produced on the farm itself and 50% of the sweet whey, in a proportion of 0.500 L, per day, by piglet and free access to water.

Treatment 4. (100%.) Balanced feed was provided, produced on the farm itself and free access to the sweet whey.

Results

weight gain

In all the treatments exists a minimum significant difference (MSD) of 0.62, corresponding to the literal (a), except the treatment 4, with a different literal (b).

At compare the average of treatments with the tabular value of Tuckey in a level of probability of $P < 0.05$ (Table 1) In the Table 1 the treatment 2 shows a further increase in weight in the last week.

TREATMENT	WEEK1	WEEK2	WEEK3	WEEK4	WEEK5	TOTAL
(T) T1 (a)	6.396	6.782	7.192	7.585	7.957	35.912 7.182
25% T2 (a)	6.89	7.179	7.46	7.762	8.128	37.419 7.484
50% T3 (a)	5.982	6.313	6.932	6.975	7.34	33.242 6.65
100% T4 (b)	6.492	6.748	7.006	7.325	7.694	35.265 7.053

Food consumption

In the treatment 1, 2 and 3 and with the same literal a minimum significant difference of 5.44 was found; not in treatment 4 with a different literal (b) in relation with the minimum significant difference, compared with the tabular value of Tuckey in a level of probability of $P < 0.05$ (Table 2).

TREATMENT	WEEK1	WEEK2	WEEK3	WEEK4	WEEK5	TOTAL
(T) T1 (a)	34.764	39.604	44.564	45.314	45.664	209.91 41.982
25% T2 (a)	32.44	31.89	32.39	36.14	41.04	173.9 34.78
50% T3 (a)	32.3	31.75	32.25	36.45	40.08	172.73 34.546
100% T4 (b)	31.35	26.65	27.95	35.8	43.3	165.05 33.01

Feed conversion

In the experimental work of replacing water with sweet whey doesn't exist a minimum significant difference of the treatment with the literal (a) that differ from the tests with the literal (b) in relation with the minimum significant difference, which corresponds to 3.17 units of conversion, compared with the tabular value of Tuckey in a level of probability of $P < 0.05$ (Table 3).

TREATMENT	WEEK1	WEEK2	WEEK3	WEEK4	WEEK5	TOTAL
(T) T1 (a)	0.359	0.386	0.41	0.393	0.372	1.92
25% T2 (a)	0.308	0.289	0.281	0.302	0.33	1.51 0.302
50% T3 (a)	0.357	0.331	0.319	0.343	0.365	1.715 0.343
100% T4 (b)	0.137	0.256	0.258	0.319	0.369	1.519 0.303

Treatments and variables

In the tabulator can be noted that doesn't exist a minimum significant difference of all the treatments, both numbered with the literal (a) as the (b) compared with the minimum significant difference of 7.93 in relation to the average of treatments tabular value of Turkey at probability level of $P < 0.05$ (Table 4).

TREATMENT	WEEK1	WEEK2	WEEK3	WEEK4	WEEK5	TOTAL
(T) T1 (a)	0	0	0	0	0	0
25% T2 (a)	23	20	20	26	31	120 24
50% T2 (b)	48	41	41	52	62	244 48.8
100% T3 (b)	103	102	106	108	125	544 108.8

Discussion

The sweet whey can be exploited to feed monogastric animals (like the pigs) mainly by its digestibility and high nutritional value as it contains essential amino acids, carbohydrates and minerals.

It's rich in water and fermentable sugars, therefore can not be stored long-term; at dispose it in drains causes pollution of rivers and lakes, so it is necessary to apply appropriate corrective measures for handling large volumes and in this way avoid problems of environmental impact.

Conclusion

It was demonstrated that the use of sweet whey in liquid form at 25% showed better performance in all parameters evaluated.

The use of sweet whey in liquid form at 25% and 50% didn't cause rejection or metabolic problems in the piglets. The piglets who received the sweet whey at the 100% had persistent diarrhea, therefore shouldn't be given to pigs this concentration of whey.

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Hepatoprotective Effect of *Picrorhiza kurroa* in Experimentally Induced Hepatotoxicity in Cockerels and Its Characterization

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Summary: Hepatoprotective properties of ethanolic and aqueous extracts of *Picrorhiza kurroa* rhizomes were evaluated in cockerels given acetaminophen @ 500 mg/ body weight orally to induce hepatocellular damage. Ethanolic extract given @ 50 mg/kg body wt and acetaminophen helped in restoration of Hb, PCV, TEC, TLC and lymphocytes and heterophils as well as total protein, albumin and globulin, glucose, cholesterol, bilirubin and activity of AST, ALT, ALP and LDH. Histopathological examination of liver section of treated birds clearly showed normal hepatic cells and central vein thereby confirming hepatoprotective activity. Silymarin used @ 200 mg/ kg body weight as reference standard also showed the same results. Aqueous extract revealed the least activity. Phytochemical analysis of ethanolic extract showed presence of alkaloids, flavonoids, glycosides, protein, resin, saponin, sterol and tannins.
Key words: *Picrorhiza kurroa*, hepatoprotective activity, histopathology, hematological biochemical profile, cockerels

Introduction

Many toxins damage the liver and affect its functions resulting in poor health and production. For prevention of hepatocytes, some drugs or chemicals are used which also antagonize the toxins and help to regain its power of metabolism. During early days, liver extract derived from liver of other mammals or fishes was the drug of choice. But such drugs posed serious risk of transmitting infections from animals to animals or to human. Moreover, the cost of liver extract is high specially if economy of the farm and farm products become a matter of concern. Now-a-day herbal liver formulations become more important in treating hepatic diseases. *Picrorhiza kurroa* has been used to treat disorders of the liver and upper respiratory tract, fevers, treat dyspepsia, chronic diarrhoea and scorpion sting [1]. *Picrorhiza* has been shown to protect liver cells from a wide variety of toxins including amanita poisoning, carbon tetrachloride [2], galactosamine [3], ethanol [4], aflatoxin-B1 [5], acetaminophen[6], and thioacetamide[7], in both *in vitro* and *in vivo* experiments. The present study was planned to investigate the activity of *P. kurroa* on liver function markers following experimentally induced hepatotoxicity in cockerel.

Material and methods

The rhizomes of *P. kurroa* procured from local market, were identified and authenticated from Department of Biological Sciences of university. These were shade dried and ground in a Willey Grinder at room temperature. For preparation of the ethanolic or aqueous extract, 100 mg each powder of *P. kurroa* was soaked in one liter of absolute ethanol or water for 48 hr at 37℃ with continuous stirring. The contents were filtered, concentrated at 45 – 50℃ and reduced pressure using rotatory vacuum evaporator [8], lyophilized to get the final extract residue and stored at 4℃ till further use. The extracts were analysed for major phytochemical groups, viz. alkaloids, anthraquinones, flavonoids, saponins, tannins, sterols, reducing sugars, glycosides, resins, triterpenes, proteins and coumarins using standard methods [9,10].

Total 100, three-month-old cockerels of same hatch were procured from IPF university and randomly divided into 5 groups Ⅰ, Ⅱ, Ⅲ, Ⅳ and Ⅴ of 20 each having almost equal average body weight and maintained under standard deep litter managemental conditions. Gr Ⅰ served as healthy control, while Gr Ⅱ received acetaminophen @ 500 mg/kg body weight orally for 7 days [11] and served as infected control. Gr Ⅲ received silymarin (as a standard reference) along with acetaminophen for 7 days and thereafter only silymarin was given upto 35th day. In Gr Ⅳ and Ⅴ, ethanolic and aqueous extract residues @ 50 mg/kg body wt [12] along with acetaminophen for 7 days and thereafter only extract were given upto 35th day. The blood samples were collected on day 0, 7, 15, 21, 28, 35 and 42 of

treatment, for haematological (Hb, TEC, TLC, PCV and DLC) and biochemical parameters (glucose, total cholesterol, total protein, albumin, globulin, albumin: globulin ratio, blood urea nitrogen and serum bilirubin and activities of enzymes AST, ALT, ALP and LDH) using standard methods. Liver samples were collected in 10% buffered formalin for histopathological examination on 7, 21 and 35 day of treatment. The results were analysed as per standard method [13].

Results and discussion

The ethanolic extract residue was greenish brown in color and oily in consistency while aqueous extract residue was light brown in color and solid dry powder in consistency. Ethanolic and aqueous extract revealed 16.09% and 13.23% yield. Phytochemical analysis of ethanolic extract of *P. kurroa* showed presence of alkaloids, flavonoids, glycosides, protein, resin, saponin, sterol and tannins, whereas alkaloids, proteins, resin and sterol were absent in aqueous extracts and anthraquinones and triterpenes were present.

There was significant decrease in Hb, PCV, TEC and lymphocytic values in group Ⅱ as compared to group Ⅰ, Ⅲ, Ⅳ and Ⅴ from 7th day onward up to the end of experiment. Ethanolic and aqueous extract, significantly restored these values to normalcy. Hb values are significantly higher in treated group than untreated and control group at 42nd day of treatment. Destruction of RBC, decrease in TEC and Hb may be due to oxidative damage-mediated removal of affected erythrocyte, induced by acetaminophen. Increased generation of free radicals can cause cell membrane damage, which in turn inactivate membrane Na^+-K^+-ATPase [14], thereby allows entry of Ca^{2+} into the cell. The sustained increase in intracellular calcium leads to free-radical generation, which in turn Na^+-K^+-ATPase. Thus the acetaminophen mediated generation of free-radicals and consequent oxidative damage to erythrocytes can cause mechanical fragility of plasma membrane, thereby shortened RBC life span and its removal from circulation. Disintegration of erythrocytes in the circulation might have resulted in reduction of haemoglobin content of blood, which in turn was associated with decrease in PCV and TEC [15]. The ethanolic extract *P. kurroa* protected the disintegration of erythrocytes. *P. kurroa* was also found to restore Na^+-K^+-ATPase levels to normal in paracetamol and aflatoxin induced hepatic injury [16]. Neutrophilia and lymphocytopenia in all the animals subjected to hepatopathy. This might be due to stress coupled with inflammatory changes in body tissue, which is responsible for phagocytosis of toxic substances and neutrophilia was induced by tissue demand for phagocytic function [17]. Administration of *P. kurroa* was also found to increase heterophils and decrease in lymphocytes [18] and restoration of TLC [19]. Glucose and bilirubin showed marked increase after induction of hepatopathy in untreated group from 7th day till end of experiment. There was significant decrease in of total protein, albumin and cholesterol levels and increase in globulin in all the treated groups than untreated group. Hyperglycaemia can be due to the degenerative hepatic lesions and also can follow the metabolic acidosis. Extracts of *P. kurroa* was also reported to reduce glucose level in blood [20]. Due to the damage of hepatocytes there was decreased elimination of bilirubin and thus an increase was observed. Many scientists observed increase in bilirubin due to hepatocytes damage [20,21]. Protein synthesized by the liver are frequently decreased in patients with liver diseases and this was manifested clinically by decrease in circulating proteins such as albumin [22]. These values came down to normalcy following therapy indicating the therapeutic values of the drug. Many scientists observed the same findings [12, 20, 23]. Hepatic cholesterol homeostasis is maintained by an equilibrium between the activities of hydroxy methyl glutaryl CoA (HMG-CoA) reductase and that of acyl CoA : cholesterol acyl transferase [24]. Reduction in cholesterol could also be due to the deficient metabolism of lipids in the liver [25]. Decrease in cholesterol level was also noticed with use of *P. kurroa* [26]. The activities of ALT, AST, ALP and LDH were elicited in infective group suggesting damage of liver hepatocytes and impairment of liver functions. Use of *P. kurroa* extracts and silymarin significantly reduced the level of these enzymes. One of the hallmark signs of hepatic injury or damage is apparent leakage of cellular enzymes into plasma [14]. These enzymes are commonly used as marker enzymes in accessing hepatotoxicity [27, 28]. Recovery towards normalization of the enzymes following *P. kurroa* treatment suggested that the plant extract have role in preserving structural integrity of hepatocellular membrane, thus prevented enzymes leakage into circulation [29,20]. There was significant decrease in feed consumption and body weight in group Ⅱ as compared to group Ⅰ, Ⅲ, Ⅳ and Ⅴ from 14th day onward till end of experiment. A significant increase in body weight was observed in the group IV at 35th day of treatment as compared to control group which might be due to increase in function of hepatocyte and increased palatability of feed. The biochemical findings were supported with histopathological observations of liver sections. The healthy control group showed normal cellular architecture with sinusoidal spaces and central veins while intoxicated cockerels revealing centrilobular hepatic necrosis. The hepatic cords were irregularly distributed and distorted and the cells were rounded with

opaque cytoplasm and showed mild vacuolated cells that suggested the fatty degeneration. In treated birds, hepitocellular changes could be restored towards normalcy.

Conclusions

These results indicated that *Picrorhiza kurroa* has hepatoprotective action. It increases the Hb, PCV, TEC, lymphoctes, total protein, albumin and globulin levels and decreases glucose, total cholesterol, bilirubin, AST, ALT, ALP and LDH values to normalcy in intoxicated bird.

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Effect of Feeding Urea-treated Sugarcane Tops on Production and Composition of Milk in Phule-Triveni Crossbred Cows

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Summary: The study was conducted in Western Maharashtra State of India on Phule Triveni lactating cows to investigate the effect of feeding a diet containing urea-treated sugarcane tops on milk yield and milk composition. Sixteen lactating cows were used and arranged in a 4 ×4 Latin square design. The daily dry matter requirement of experimental cows was fixed at 3% body weight of the cows and the treatment diets consisted of concentrate and roughage at a proportion of 1/3 and 2/3 part of the ration (dry matter basis), respectively. The concentrate portion of the diet was similar for all treatments. The roughage dry matter proportion of the treatments were T_0 (30% untreated sugarcane tops, 20% green maize, 30% green lucerne and 20% sorghum straw), T_1 (30% from 4% urea-treated sugarcane tops, 20% green maize, 30% green lucerne and 20% sorghum straw), T_2 (45% from 4% urea-treated sugarcane tops, 15% green maize, 20% green lucerne and 20% sorghum straw) and T_3 groups consisted of 60% from 4% urea-treated sugarcane tops, 20% green lucerne and 20% sorghum straw. There was no significant difference ($P > 0.05$) among treatments in respect of milk yield and milk constituents (protein, fat, TSS, SNF and calcium). Diets with 45% UTSCT (T_2) and control group (T_0) were the best and the least treatments, respectively in respect of economic profitability. Higher net income was observed in all urea-treated sugarcane top based diets than the control diet. It can be concluded that urea-treated sugarcane tops can be incorporated up to 45% of the roughage requirement in the diet of lactating cows for better milk production and profitability with out affecting the milk composition.
Key words: milk production, milk composition, sugarcane top, urea-treatment

Introduction

Sugarcane top is a major by-product of the sugar industry which is often left in the field unutilized after harvest. The sugarcane top consists of 3 distinct parts: the green leaves (blades), the leaf sheath bundle and variable amount of immature cane. The yield of tops varies considerably with variety, age at harvest, growing condition and management practices [1].

Sugarcane tops silage enriched with urea and molasses could be used in dairy cattle nutrition and replace up to 75% forage ration without any negative productivity effects instead of Lucerne [2]. Similarly, research findings indicated that feeding of treated sugar cane top silage treated with 1% urea and 3% molasses can replace up to 100% of maize silage in rations for milch buffaloes with out any effect on intake, milk production and composition [3]. Thus, sugarcane top is extensively utilized as animal feed in Maharashtra state. However, there is dearth of information available on the influence of feeding urea-treated sugarcane top on production and composition of milk in lactating cows. Therefore, this study was undertaken to investigate the effect of feeding urea-treated sugarcane top based diet on milk yield and its composition in crossbred cows.

Material and methods

The study was conducted at Research Cum Development Project (RCDP), which is located 160 km North East of Pune city, Marashtra State, India. The area lies at 569 m above sea level on the 18°32′ North latitude and 73°51′ at east longitude. The area has a bimodal pattern of rainfall and it receives an annual rainfall of 530.5 mm. The average maximum (39℃) and minimum (10.8℃) temperatures occur in May and December, respectively.

The experimental feed consisted of concentrate and roughage in 1 : 3 (DM basis) [4]. The concentrate portion of the diet was similar for all treatments. The roughage dry matter proportion of the treatments were T_0 (30% untreated sugarcane tops, 20% green maize, 30% green lucerne and 20% sorghum straw), T_1 (30% from 4% urea-treated sugarcane tops, 20% green maize, 30% green lucerne and 20% sorghum straw), T_2 (45% from 4% urea-treated sugarcane tops, 15% green maize, 20% green lucerne and 20% sorghum straw) and T_3 groups consisted of 60% from 4% urea-treated sugarcane tops, 20% green lucerne and 20% sorghum straw.

The urea treatment of sugar cane tops was done in underground concrete silo pit which had a capacity of treating 15 tones. Four kg urea fertilizer was dissolved in

50 litres of clean water for 100 kg sugarcane tops on dry matter basis. The silo pit was filled and the stack was covered tightly with a plastic sheet to exclude the entrance of oxygen and prevent ammonia loss. The treated sugarcane top was ensiled for three weeks before opening for feeding.

Sixteen lactating Phule Triveni triple crossbred (50% Holstein Friesian + 25% Jersey + 25% local *Gir*) cows aged 4.6 ± 1.2 years were used. The cows had 8.97 ± 2.4 litres and 395.9 ± 53.4 kg of initial milk yield per day and body weight, respectively. The experiment was started after the cows passed 145 ± 37.1 days in lactation. The animals had 10 days of adaptation period and the feeding trial carried out for 90 days.

During the entire period of the experiment, samples of offered and refusals (left over) were taken on weekly interval and bulked for the entire period of the experiment and were analyzed for dry matter (DM), ash, crude protein (CP), ether extract (EE) and crude fibre (CF) content according to the standard method [5].

Fresh milk samples were collected at monthly interval from each experimental cow from morning and evening milking. Composite morning and evening milk samples were kept overnight in a refrigerator maintained below 4℃ before undertaking the required test for chemical composition. Milk fat analysis was run using the Gerber method [6]. Protein content was determined by using the formaldehyde titration method [7]. Total solids (TS) were determined by oven drying 5 g of milk [8]. Solid not fat (SNF) was calculated by subtracting fat per cent from total solid. Milk calcium was analyzed according to the standard method [9].

Milk urea was determined at 75th and 90th days of the experiment by using p-dimethyl amino benzaldehyde reagent [10].

The economic evaluation was based on computation of the total cost incurred for milk production and the amount of benefit gained from the sale of milk. The milk price was computed at Indian rupee (INR) 12/litre, which was the official price at RCDP.

A 4 ×4 Latin Square design that comprised four age groups and four stages of lactation (days in milk) was employed. Daily milk yield, milk fat, milk protein, SNF, total solids, milk calcium, and milk urea were analyzed using Proc ANOVA of the SAS System for Windows [11].

Results and discussion

Milk Yield and Milk Composition

The average daily milk yield and milk composition of the experimental cows in respective to their treatment groups are presented in Table 1.

Table 1 Effect of experimental diets on milk yield and milk composition

Parameters	Experimental diets (treatments)				Overall mean	SE
	T_0	T_1	T_2	T_3		
Milk yield($l \cdot d^{-1}$)	8.10	8.88	9.20	8.43	8.65	1.29
Milk composition Protein(%)	3.60	3.35	3.36	3.44	3.44	0.07
Fat(%)	3.90	3.98	3.90	3.80	3.90	0.16
TS(%)	13.37	13.06	13.78	13.43	13.41	0.32
SNF(%)	9.48	9.11	9.91	9.63	9.53	0.33
Calcium ($mg \cdot l^{-1}$)	1105.49	1224.23	1155.74	1153.55	1159.75	42.01
Urea ($mg \cdot dl^{-1}$)	40.57^{c}	66.79^{b}	73.80^{ab}	80.52^{a}	65.42	2.56

^{a,b,c} = means in a row which having different superscripts are significantly different at $P < 0.05$.

As shown in Table 1 there were not significant differences among treatments in terms of milk yield and milk constituents except milk urea. The research conducted on feeding of different levels of urea-treated sugarcane tops silage had shown non-significant effect on milk production, milk fat, milk protein and TS [2]. Similarly, the study carried out on feeding of different amounts of urea-treated sugarcane tops on buffalo resulted non-significant variation in milk production and milk composition [3].

Economic Analysis

According to the cost-benefit analysis calculated for each diet, the highest and lowest cost of production was observed in T_2 (INR 60.91 $cow^{-1} \cdot d^{-1}$) and T_3 (INR 58.31 $cow^{-1} \cdot d^{-1}$), respectively. The net income from cows for treatment T_2 (diet with 45% UTSCT) was highest as compared to other treatments and all of the urea-treated sugarcane top based diets were higher in net income (INR 46.80, 49.49, 42.49 $cow^{-1} \cdot d^{-1}$ for treatment groups of T_1, $T_2$and T_3, respectively) than the control (T_0) treatment (INR 38.20 $cow^{-1} \cdot d^{-1}$). The highest and lowest value of benefit to cost ratio was noticed in T_2(1.81) and T_0(1.65), respectively. Diets with 45% UTSCT (T_2) and control group (T_0) were the best and the least treatments, respectively in respect of economic profitability.

Conclusions

It can be concluded that urea-treated sugarcane tops silage can be incorporated up to 45% of the roughage

requirement in diet of lactating cows for better milk production, feed utilization and profitability with out affecting the milk composition of cow.

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Isoflavone and Flavanones as a Potential Dietary Strategy to Improve the Health Performance of Broiler Chickens

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Summary: Flavonoids have been recognized as a health promoting phytochemicals in several animal studies. Among different flavonoids sub-classes, isoflavone and flavanone are known to be important due to their multidimensional health effects. In present study an isoflavone genistein and a flavanone hesperidin were observed individually and in combination for health promoting effects in broiler chickens. One-day-old broilers (n = 360) were divided into six treatment groups and offered corn-soybean basal diet with supplementation of: CON (no additive), G5 (5 mg genistein kg^{-1} feed), and H20 (20 mg hesperidin kg^{-1} feed); while, other three groups were supplemented with a mixture of genistein and hesperidin (1∶4) having dosage of 5 $mg \cdot kg^{-1}$ (GH5), 10 $mg \cdot kg^{-1}$ (GH10) and 20 $mg \cdot kg^{-1}$ (GH20) diets, respectively. Chickens were slaughtered at 42 and indices of spleen, thymus and bursa were calculated. Samples including liver and pectoralis major muscle were collected and analyzed for antioxidative parameters including, total antioxidant capacity (TAOC), malondialdehyde production (MDA) and total superoxide dismutase (SOD) activity. Blood samples were also collected from growing broilers at 20, 27, 34 and 42 d to investigate the antibody titers against Newcastle disease (ND) and avian influenza (AI) disease. Results reveled that, isoflavone genistein and flavanone hesperidin could significantly improve the immune organ indices ($P < 0.05$), antioxidative activity ($P < 0.05$) of liver and pectoralis major and antibody titers ($P < 0.05$) against ND and AI diseases. Effects of combinatorial treatments of genistein and hesperidin were observed better as compared to their individual treatments. These results indicate the potential of isoflavones and flavanones to improve the health of farm animals including broiler chickens.

Introduction

Poultry industry is highly dependent on the antibiotic feed additives to maintain the health and productivity of commercial chickens; however, currently these antibiotics have been banned by the health regulatory authorities [1]. In recent years, considerable interest has been shown on research of alternative feed additives including prebiotics, probiotics and phytogenic additives like flavonoids to improve the health performance of farm animals. In contrast to antibiotics these products do not have any side effects and are thus considered safe in food chain.

Flavonoids, a group of plant polyphenols, have been recognized as health promoting compounds in several animal studies. Among several flavonoid subclasses, isoflavone and flavanone are known to be important due to their multidimensional health effects. Both isoflavone and flavanone are abundantly found in soybean and citrus fruits respectively. In human studies, these have been reported as antioxidant, immunostimulant, anti-inflammatory, anticancer and cardioprotective; thus regarded as health-promoting dietary compounds' [2]. From available *in vivo* studies, it is clear that isoflavone and flavanone are natural antioxidative and immunomodulatory agents that could stimulate the T lymphocytes; and increase the antibody production and relative weights of immune organs [3,4]. These could affect the body's redox balance, decreases oxidative damage to lipids, and improves the antioxidant status of individuals [5]. In present study, an isoflavone genistein and a flavanone hesperidin were investigated individually and in combination (1∶4) for their health effects including immune organ indices, antibody titers and antioxidative effects in broiler chickens.

Material and methods

A total of 360, day-old broiler chicks (Arbor Acres) were purchased from a local hatchery and were divided into 6 treatment groups, 6 replicates of 10 birds each. These were assigned to wire cages and housed in an environmentally controlled room. Broilers were allowed to consume both feed and water ad libitum. Broilers were offered a corn-soybean basal diet with supplementation of: CON (no additive), G5 (5 mg genistein kg^{-1} feed), and H20 (20 mg hesperidin kg^{-1} feed); while, other three groups were supplemented with a mixture of genistein and hesperidin (1∶4) having dosage of 5 $mg \cdot kg^{-1}$ (GH5), 10 $mg \cdot kg^{-1}$ (GH10) and 20 $mg \cdot kg^{-1}$ (GH20) diets, respectively. The diets were formulated according to stages of growth, i-e starter and grower. Genistein and hesperidin were purchased from a commercial source (Sigma Chemical Co. USA) with 98% purity.

At 20, 27, 34 and 42 d of age, individual blood samples were collected from wing veins and analyzed for anti-ND and anti-AI antibody titers through haemagglutination inhibition assay [6]. The results of HA/HI were expressed as the geometric mean titer (GMT) of log2. At 42 d of age, broilers were deprived off feed for 12 h and 2 broilers per replicate were randomly selected, weighed and sacrificed. Liver and meat (pectoralis major) samples were collected for antioxidation and analyzed through commercially available kits (Nanjing Jiancheng Bioengineering Co., China). Immune organs including, thymus, bursa and spleen were collected and weighed to calculate their indices (relative weights). All the statistical analyses were done by using ANOVA through JMP statistical package software (Version 5.0.1.a, SAS Institute. Inc. Cary, NC, USA) and results were presented as means SEM (standard error of mean).

Results and discussion

Immune organ indices

As shown in Table 1, the thymus index was found to improved ($P < 0.05$) by the dietary treatments of flavanone and isoflavone. However, in comparison with control group no group has showed the significant difference. The indices of bursa and spleen were remained unaffected ($P > 0.05$) by the treatments of flavonoids. These results could be associated with a recent study of flavonoid-rich extract [7] that significantly increased the size of thymus, and suggested the immunomodulatory potential of flavonoids.

Table 1 Effect of dietary isoflavone and flavanone on immune organ indices of broilers

Parameters	Treatments						P value
	CON	G5	H20	GH5	GH10	GH20	
Thymus index	0.351 ± 0.009^{ab}	0.396 ± 0.017^{ab}	0.370 ± 0.020^{ab}	0.349 ± 0.009^{b}	0.410 ± 0.013^{a}	0.411 ± 0.013^{a}	0.008
Bursa index	0.140 ± 0.005	0.162 ± 0.032	0.193 ± 0.016	0.133 ± 0.005	0.173 ± 0.004	0.180 ± 0.003	0.070
Spleen index	0.132 ± 0.007	0.142 ± 0.011	0.153 ± 0.013	0.150 ± 0.019	0.161 ± 0.012	0.158 ± 0.009	0.331

Each value represents the mean SEM; n = 12

^{ab} Mean values in a row that not sharing a superscript are significantly different ($P < 0.05$).

Antibody titers

Serum anti-ND titer was found elevated ($P < 0.01$) for all combined supplemented groups i.e., GH5, GH10 and GH20 groups in comparison with control group on 20 d (Table 2), while anti-AI titer also increased significantly ($P < 0.01$) for H20, GH5, GH10 and GH20 groups. On 27 d, antibody titer against ND was improved ($P < 0.01$) for all treated groups, while antibody titer against AI was also increased ($P < 0.01$) for all groups except G5 group, in comparison with control. On 34 d, antibody titer against ND and AI was improved ($P < 0.05$) only for GH20 group, in comparison with control group. On 42 d, serum anti-ND titer increased significantly ($P < 0.05$) for G5 and GH20 groups while no change ($P > 0.05$) was observed in anti-AI antibody titer.

The present study indicates that isoflavone and flavanone were evidently immunogenic for to improve antibody titers against ND and AI diseases. One of the possible explanations for this is the antioxidative activities of these compounds [5], because T-and B-cell-based immune reactions are highly sensitive to oxidative damage [3]. Hanieh et al. reported that purple sweet potato powder with 38% flavonoids could modulate the antibody titer of chickens in dose-dependent fashion [6].

Table 2 Effect of dietary isoflavone and flavanone on serum anti-ND (Newcastle disease) and anti-AI (avian influenza) antibody titers of broilers from 20 to 42 d of age

Parameters[1]	Treatments						P value
	CON	G5	H20	GH5	GH10	GH20	
Anti-ND							
20 d	3.833 ± 0.30^{c}	4.833 ± 0.30^{bc}	5.333 ± 0.42^{abc}	5.667 ± 0.33^{ab}	6.667 ± 0.42^{a}	6.667 ± 0.33^{a}	< 0.001
27 d	4.167 ± 0.31^{b}	6.0 ± 0.57^{a}	7.0 ± 0.36^{a}	6.833 ± 0.47^{a}	7.333 ± 0.21^{a}	7.333 ± 0.42^{a}	< 0.001
34 d	5.333 ± 0.33^{b}	6.667 ± 0.42^{ab}	6.50 ± 0.56^{ab}	5.833 ± 0.30^{b}	6.333 ± 0.33^{ab}	7.833 ± 0.16^{a}	0.001
42 d	5.667 ± 0.21^{b}	7.0 ± 0.36^{a}	6.667 ± 0.21^{ab}	6.667 ± 0.33^{ab}	6.667 ± 0.33^{ab}	7.333 ± 0.21^{a}	0.008
Anti-AI							
20 d	4.0 ± 0.25^{c}	4.667 ± 0.33^{bc}	6.0 ± 0.36^{ab}	6.0 ± 0.36^{ab}	6.50 ± 0.34^{a}	7.0 ± 0.44^{a}	< 0.001
27 d	5.167 ± 0.30^{bc}	5.0 ± 0.25^{c}	6.50 ± 0.34^{ab}	6.833 ± 0.40^{a}	7.0 ± 0.25^{a}	7.333 ± 0.42^{a}	< 0.001
34 d	5.333 ± 0.66^{b}	6.167 ± 0.60^{ab}	6.0 ± 0.51^{ab}	6.333 ± 0.21^{ab}	6.50 ± 0.42^{ab}	7.833 ± 0.16^{a}	0.023
42 d	6.50 ± 0.42	7.0 ± 0.36	6.667 ± 0.49	6.50 ± 0.34	6.6667 ± 0.42	7.0 ± 0.46	0.912

Each value represents the mean ± SEM; n = 12

^{abcd} Mean values in a row that not sharing a superscript are significantly different ($P < 0.05$).

[1] expressed as geometric mean titer (log2).

Antioxidative effects

The total antioxidant capacity (TAOC) of pectoralis major muscle and liver were raised ($P < 0.01$) by the isoflavone and flavanone treatments, however, in comparison with control group no group has changed the TAOC level (Table 3). The superoxide dismutase activity (SOD) of pectoralis major was significantly improved ($P < 0.05$) in all treated groups except for G5 group, while the SOD activity of liver raised in H20 and GH20 groups as compared to control group. The malondialdehyde (MDA) contents of pectoralis major were significantly decreased ($P < 0.01$) in all combined supplemented groups, whereas, the MDA contents of liver were reduced in H20, GH10 and GH20 groups in comparison with control group. These results indicate that flavonoids could inhibit the lipid oxidation and also enrich the poultry products (meat and liver) for antioxidants. Previous studies have also acknowledged the potential of phytochemicals to improve the oxidative stability of poultry meat and also improved antioxidative status that may lead to enhanced health performance and productivity [8].

Table 3 Effect of dietary isoflavone and flavanone on TAOC (U/mg of protein), SOD (U/mg of protein) and MDA (nmol/mg of protein) levels of liver and pectoralis major muscle in broilers

Parameters[1]	Treatments						P value
	CON	G5	H20	GH5	GH10	GH20	
Pectoralis major							
TAOC	1.210 ± 0.042^{acd}	1.126 ± 0.021^{d}	1.291 ± 0.028^{bc}	1.316 ± 0.012^{abc}	1.333 ± 0.009^{ab}	1.421 ± 0.030^{a}	<0.001
SOD	42.51 ± 1.16^{d}	48.91 ± 1.21^{cd}	45.37 ± 1.18^{c}	55.76 ± 1.18^{b}	57.13 ± 1.15^{b}	63.09 ± 1.9^{a}	<0.001
MDA	1.572 ± 0.18^{a}	1.462 ± 0.09^{a}	1.411 ± 0.02^{a}	0.921 ± 0.04^{b}	0.767 ± 0.02^{b}	0.724 ± 0.01^{b}	<0.001
Liver							
TAOC	2.45 ± 0.17^{ab}	2.17 ± 0.30^{b}	2.85 ± 0.02^{a}	2.75 ± 0.09^{ab}	2.81 ± 0.04^{a}	2.92 ± 0.02^{a}	0.008
SOD	266.4 ± 9.33^{b}	279.9 ± 9.96^{ab}	303.6 ± 9.48^{a}	283.4 ± 10.26^{ab}	296.5 ± 5.47^{ab}	309.0 ± 6.09^{a}	0.013
MDA	2.12 ± 0.03^{a}	2.04 ± 0.02^{a}	1.83 ± 0.03^{b}	2.05 ± 0.02^{a}	1.88 ± 0.02^{b}	1.84 ± 0.01^{b}	<0.001

Each value represents the mean SEM; n = 12

[abcd] Mean values in a row that not sharing a superscript are significantly different ($P < 0.05$).

[1] TAOC = total antioxidant capacity; SOD = superoxide dismutase activity; MDA = malondialdehyde contents.

Conclusions

This study suggested that isoflavone genistein and flavanone hesperidin could improve the immune organ indices, serum antibody titer and antioxidative status of meat and liver in broilers. Thus, these could be used as alternative of synthetic feed additives in poultry industry to improve the heath performance of broiler chickens.

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Livestock, Resources and Human Demand: The Sustainability Debate

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Summary: Sir John Beddington (the UK's Chief Scientific Adviser) argues for more sustainable livestock as one element of a new "green*er* revolution" in food production (Beddington 2009). Development of sustainable livestock farming practices appears to fall into two categories: sustainable intensification or a reduction in demand for meat, eggs and milk through modification of consumer behaviour. This paper examines global demand for animal products, which is forecast to increase as a result of global population growth and the emergence of an increasingly affluent middle class in developing countries, and the associated issue of bioethics. However, there is currently little comparative evidence to indicate that one is more "sustainable" than the other in terms of their impact on production systems, usage of global supplies of energy and water, potential contributions to maintaining human health and meeting future demand for food.

Introduction

Expanding demand for farm animal products is inherently unsustainable for several reasons, including greenhouse gas emissions, particularly from ruminants; standards of farm animal welfare and health, especially when animals are kept intensively; poor efficiency of conversion by livestock of solar energy into food; water usage in livestock production and the growing evidence of the adverse implications for human health through the consumption of meat. Solutions must be found if the Perfect Storm of global events forecast by Beddington is correct, as within this context of climate change and a global human population of c. 9. 5 billion by 2030, failings in food, energy and water security are more likely.

Broadly speaking, consumer demand for meat, eggs, milk and other animal products is forecast to rise over the coming 20 – 30 years, in line with an exponential rise in global population (e. g. Royal Society 2012), whilst the supply of inputs e. g. animal feeds and water is likely to fall, despite technological advances. Pressure on dwindling resources will, under economic laws of supply and demand, result in market uncertainty and/or increasing worldwide food prices for many types of food, including staples (a trend that has been noted during 2012). These trends could accelerate if Beddington's Perfect Storm concept does occur in c. 2030.

We wish to make two key points in this discussion paper. First, we maintain that concerns about food security and sustainability impact differentially upon livestock systems. Diets of pigs, chickens, other poultry and cattle house all-year-round are largely made up of cereals, pulses and other grains that could be consumed directly by humans, who prefer to eat pork, ham, sausages, chicken breast and eggs to (raw) plant ingredients. Feeding higher up the food chain is a characteristic of an affluent society. Some object strongly to this practice while others allow such consumption in a free-market, subject to certain limitations, e. g. statutory minimum standards of farm animal welfare. It has become commonplace to talk about livestock' and livestock systems' in a generic fashion (for example, FAO, 2006). However, much more work is needed to model and calculate the impacts of the two systems that are emerging as front runners in the sustainability debate: sustainable intensification or reducing overall demand for meat, eggs and dairy products through modification of consumer behaviour.

Secondly, we propose that a notion of sustainability that does not take into account the quality of an animal's life is unethical. Arguments for sustainable intensification, for example, should consider the implications for animal welfare of sustainable technologies and practices. We argue that there is a real danger that the animal welfare gains achieved over the past 40 years may be lost under expected economic pressures of future human food security issues.

Beddington's perfect storm

Beddington argues that globally, by 2030, 50% more food and energy and 30% more fresh water will be needed for the human population, but utilising less land, pesticides, fertilisers and water. We will not know whether his forecast of a Perfect Storm is correct until c. 2025 at the earliest; it may simply be a worst-case scenario. Nonetheless, UK supermarkets are incorporating its likely

effects into their medium-and long-term planning forecasts, whilst they develop increasingly stringent animal welfare standards due to consumer demand. Moreover, greater competition between energy and food markets will occur over land for livestock and crops (Mitchell 2008), as a result of both biofuels being used for transport and biomass for heat and electricity to meet global energy demands: projected to increase by 45% between 2006 and 2030 (IEA 2008).

Worldwide demand for livestock food products is strong and growing, especially in the newly advanced economic development countries of Brazil, Russia, India and China (BRIC), where the rapid growth of an affluent middle class is accompanied by a growing demand for animal protein. From 2004 – 2006 to 2010, gross production per capita of livestock changed by – 2.6% and +12.2% in the UK and China, respectively (FAO, 2012). Popkin (1994) has highlighted the irony that he terms the "Nutrition Transition" whereby an increased demand for meat by mainly middle class consumers can result in the paradox of under-and over-nutrition in the same locale.

There is also increasing disquiet about both increased antibiotic resistance and livestock-derived zoonoses such as avian flu (especially through intensive livestock production systems), as well as the adverse impact of a diet rich in animal-based food on human health (e.g. Donaldson, 2004). Adoption of Western diets by millions of people in developing countries may have adverse consequences for human health and associated escalating costs to health economies through the rising incidence of some cancers, dementia, obesity, diabetes and cadiovascular disease (e.g. Cordain et al., 2005).

Significant concerns accompany this unparalleled growth being so heavily dependent upon animal, rather than plant, products. Many criticize livestock farming because of the inherent inefficiency with which water, grass, grain and other feedstuffs are transformed into food. For example, it can take 1,000 l of water to produce 1 kg of milk and 15,500 l of water to produce 1 kg of beef (Hoekstra, 2010). Consumption by livestock of soya beans is closely associated with the loss of mostly South American rain forest, plus heightened emissions of CO_2, CH_4 and other green house gases, dust and NH_3, and an imbalance of omega 6∶3 fatty acid ratios post-ingestion in comparison with animals sustained on food they evolved to consume (Daley et al, 2010). Alongside moral critiques of meat consumption *per se*, there is a growing ethical concern for the quality of life of farm animals linked to certain husbandry systems.

Sustainability

The scale of use of farm animals in Britain has grown substantially over the past few decades, such that annually, nearly a billion farm animals are reared in the UK, the majority of which are broiler chickens kept for meat, which are nominally protected in law from extremes of suffering. An acceptable level of necessary suffering" is a critical area for social debate, ethical engagement, economic argument and scientific research. Whether livestock should be used in such a way is beyond the scope of this paper (such ethical questions were asked at the First International Conference on Veterinary and Animal Ethics held in 2011 (see Wathes et al., 2011)).

What does sustainable livestock production actually mean? Should animal welfare be an integral part of it's definition—or not? Traditionally, sustainability has been defined as a balance between economic, environmental and social issues, of which farm animal welfare may be considered a component of the third; linked with bioethics and societal recognition of farm animals as sentient beings. Others consider welfare as principally a critical part of economic sustainability, either through a concern for productivity, the welfare of farm animals as economic units or a concern for the social value of providing additional welfare as a costly public good one that increasing numbers of UK consumers appear willing to pay, evidenced by the increasing animal welfare standards required by UK supermarkets by 2020 and increased sales of food produced of known welfare provenance, for example the RSPCA's "Freedom Foods" range (RSPCA, 2008). Others argue that sustainability is too implicitly anthropocentric and that animal lives should constitute a fourth, separate pillar within the sustainability framework.

Sustainable intensification

Sustainable intensification links greater concentrations of livestock with mechanised production systems in a bid to meet a growing global population's demand for more meat, eggs, milk and other livestock products. There is a growing recognition that it will be difficult to meet this demand without enhanced, integrated resource management including agriculture and careful consideration of the implications for food policy. We argue that in pursuit of sustainable intensification, food chain actors, resource managers and agricultural policy-makers must not repeat the mistakes of previous waves of intensification in agricultural and food policies.

The UK Foresight Report (2011) promotes "sustainable intensification" as the agricultural solution to the many perils awaiting humanity in c. two decades time. The phrase appears to be the new buzzword in global agri-

cultural development, at least amongst many Western countries. Of course, intensification can mean different things. Inputs can be intensified and concentrated, whether they be artificial or organic inputs into cultivation of the more traditional inputs of human economic endeavour, e. g. land, labour and capital.

However, in the context of livestock farming, intensification usually refers to the increasing intensity, concentration and relative length of time that animals are housed—with the most intensive systems meaning both all-year-round housing and zero-grazing. We ask whether these should be considered criteria for sustainability? Developed primarily as an objective for arable farming, the UK's Farm Animal Welfare Committee (2012) has argued that sustainable intensification *per se* is an oxymoron when applied to livestock, questioning its ability to capture the complex requirements when animals are farmed, e. g. the consequences of increased "metabolic stress", the use of often globally-transported alternative fodder and a "relative decline in health and welfare" (Huxley 2011). Our point here is that to develop a sustainability policy without accounting for the animal welfare impact of that policy and doing everything possible to ameliorate adverse effects of intensification on animal welfare (or the environment) is unethical.

Critics of sustainable intensification as a solution to the many perils following a Perfect Storm point to a number of other issues:

· The economic impact of large-scale livestock units on small farms

· Amenity issues

From our point of view, livestock agriculture cannot approach the notion of sustainability if an animal's life is not worth living, with the attendant danger that the term 'sustainable intensification' becomes the new hegemony, thus making it difficult for alternative systems and arguments to flourish. Pursuit of sustainable intensification should not be promoted at any cost.

Other solutions to beddington's perfect storm

However, numerous studies (e. g. Pond et al., 2011) have demonstrated the significant welfare disadvantages that can sometimes result from poorly managed, free-range systems, despite the term evoking benign connotations of naturalness. Therefore, unequivocal advocacy of production systems that necessarily equate outdoor access, however brief, as a universal welfare panacea and inherent component of sustainable livestock production should be treated with caution.

On the one hand, population growth, affluence and global economic liberalism are driving what some have referred to as a neo-productivist paradigm in agriculture, i. e. a global rush to produce even more meat, eggs and milk from rapidly growing livestock, with ever higher inputs to improve efficiency of it's production. Another school maintains that only by reducing the demand for meat can the global human population be fed sustainably in the future-i. e. reducing the proportion of meat on the dinner plate or making meat and other livestock products more of an 'one-off' foodstuff than a staple (D'Silva and Webster, 2010) although how this is to be achieved on a national or global basis under different social and cultural systems is open to question. Alternative arguments include a reduction in global human population and other solutions being investigated include increasing the use of insects for food and consumer acceptance of *in vitro* or imitation meat products.

Despite the inefficiency with which farm animals transform solar energy, some argue that only meat and milk from ruminants should be encouraged as pasture on hills and uplands yields grass that can only be converted by ruminants into food for humans. Others do not accept this argument, even when land is unsuitable for grain production because of its slope and other topographical features, because there are environmental concerns regarding these delicate ecosystems.

What is sustainable livestock production? We believe that more research is needed into the following questions: do any of the possible solutions to the sustainability debate contribute or ameliorate the drivers of Beddington's Perfect Storm? Do any potentially improve human health or minimise the number of humans who will go hungry? Will any maximise destruction of rainforests and other natural habitats or minimise the release of global gas emissions, thus helping to meet the need to both mitigate climate change and to adapt to it considering Beddington reports that global greenhouse gas emissions must be reduced by at least 50% – 60% by 2050 compared to current levels. Will any minimise competition between energy, water and food markets in comparison with other identified potential solutions? Is high welfare vs. standard welfare meat production more, or less, "sustainable" under any of these parameters?

The implications of all potential solutions to Beddington's Perfect Storm should be modelled for their consumption of global supplies of energy and water, their potential contribution to the demand for food and health resources as well as their impact on production systems in both developed and developing countries.

Conclusions

Sustainability, livestock production, human health and natural resource management have been debated for over 40 years but the complex, multi-factorial linkages between them—and the inclusion of both human and

animal ethics-are only recently beginning to be investigated through a collaboration of experts across the spectrum of academic specialties. It is therefore difficult to draw conclusions, expecially before further calculations on the capabilites of the ability of either sustainable intensification or eating less meat/animal produce through behaviour modification at different population totals are finalised—or even before we know whether the forecast of a Perfect Storm is accurate.

Expansion of natural and water accounting frameworks is encouraging, whereby the inclusion of natural capital in economic and business decisions could strengthen sustainable livestock production and could be useful if utilised with collaborative, transnational food policies within a rational natural resource management programme. These include the Triple Bottom Line (Elkington, 1994) which incorporates social, environmental (or ecological) and financial dimensions of performance to which it can be difficult to assign appropriate means of measurement; the UK's suggested global green accounting system, "GDP-plus" (Defra, 2012) and the World Bank's 50/50 campaign (2012), which formally put a value on environmental assets as much as economic output.

We accept that it is possible to discuss all these developments with no reference to animal welfare but have argued that this is unethical. What is becoming clear is that these interlinked and related topics will determine human health and hunger over the coming decades, as well as what class(es) of farm animal are required to provide food for human consumption and other livestock products. Leadership, international collaboration and political determination will be required, rather than hoping that breakthroughs in engineering technology coupled with market forces will solve our sustainable resource problems: unless, that is, we are overtaken by the Perfect Storm first.

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Hygienic Sanitary and Chemical Composition Estimation of Maize Silage in Dairy Farms in Lithuania

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Abstract: The aims of this study were to estimate maize silage hygienic sanitary and chemical composition parameters, contamination with mycotoxins in dairy farms in Lithuania.

In 2011 – 2012 were collected maize silage samples from 20 dairy farms: prior to ensiling and after 3 and 8 months of ensiling.

The method used to detect of *L. monocytogenes* from raw and ensiled maize silage - LST ISO 11290-2:1998. For identification of yeasts, moulds, total of microorganisms count were used direct plating and dilution plating. Mould genera and species were estimated PCR and microscopy methods. Contamination with total aflatoxins (AFL), deoxynivalenol (DON) zearalenone (ZEN), T-2 toxin and ochratoxin A was tested with the RIDASCREEN test kits. The estimation chemical composition and digestibility parameters of corn silage were used near infrared reflectance spectroscopy.

Before ensiling in maize was detected 25.0% *L. monocytogenes*, after 3 month of ensiling 2%. Average of total count of microorganisms and yeasts in raw material samples respectively 122.2 and 71.39 cfu/g, after three months 189.01 and 71.39 cfu/g, after eight months 258.7 and 213.81 cfu/g. In maize was dominated *Fusarium* spp. and in silage - *Alternaria*, *Aspergillus*, *Cladosporium*, *Penicillium* spp.

Compared raw and after three months of ensilage maize samples, higher mycotoxins concentrations were detected in samples after ensilage: AFL – 94.42% ($P < 0.05$), DON – 36.96% ($P > 0.05$), ZEN – 77.32% ($P < 0.05$), T-2 toxin – 71.58% ($P < 0.05$). Ochratoxin A concentration was detected in maize silage after 3 months of ensiling – 48.58 ± 2.17 μg/kg and after 8 months of ensiling – 35.08 ± 5.55 μg/kg.

Dry matter and pH respectively: prior to ensiling 36.96 and 5.26%, after 3 month of ensiling 33.17 and 3.84%.

The main finding of this study is that maize silage hygienic sanitary parameters change for the worse after long period of ensilage; changes of chemical parameters are insignificant.

Key words: microorganisms, moulds, mycotoxins, maize silage

Immunotoxico-pathological Alterations Rendered by Chlorpyrifos in Broiler Chicks

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Abstract: Chlorpyrifos (CP) is an amber to white, crystalline solid, moderately soluble in water organophosphate insecticide, acaricide, and nematicide. It is used for treatment of dog kennels, and for domestic dwelling, farm buildings, and control of termites in chicken houses. Despite the restriction of its domestic and agricultural uses, CP still remains one of the most widely used. The present study was aimed upon the determination of immunotoxico-pathological effects of chlorpyrifos (CP) in 145 a-day-old broiler chicks. Birds were divided into five equal groups. First three groups, i. e., CP-5, CP-10 and CP-15 received CP diluted in xylene @ 5, 10 and 20 mg/kg b. wt orally daily for 15 days, respectively. Xyl group received xylene alone @ 1 mL/kg b. wt, while control group physiological saline solution. Randomly selected five birds from each group were killed humanly at day 0, 15, 30 and 45 for blood and morbid tissues (of liver, lungs and kidneys) collection for serological and histopathological studies.

The clinical signs observed included depression, dullness, weakness, salivation, lacrimation, gasping, frequent defecation, tremors, convulsions and drooping of wings. Feed intake and body weights were also decreased. Absolute weight of liver and kidneys was increased in treatment groups at the end of the experiment as compared to control group. Absolute weight of intestines, spleen and bursa of Fabricius was decreased in treatment groups as compared with control group. Humoral immune response was examined by antibody response to sheep red blood cells. Cell-mediated immunity was measured using lymphoproliferative response to avian tuberculin and phagocytic ability was measured by using carbon clearance assay. Results showed that CP administered broiler chicks exhibited a dose-dependent decrease in humoral immunity, cell mediated immunity and phagocytic ability was observed in chicks administered with CP. From the present study it was concluded that CP is proficient of inducing immunotoxic alterations.

Feed Additives and Nutrition

Milk Production and Some Blood Metabolites of Egyptian Buffaloe Cows as Affected by Chromium Methionine Supplementation

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Summary: The experiment was conducted to study the effect of Chromium supplementation on performance and blood serum biochemistry of lactating buffaloe cows. Therefore, thirty lactating buffaloes (aged 3 – 6 years and aver. B. Wt 567 ± 22.78 kg) were equally divided into two groups. Group one (G1), Control, which did not receive Chromium in their rations and group two (G2), treated, received 5 mg/head/day of Chromium methionine in their rations from 6 – 19 WKs postpartum.

Milk production and milk composition were evaluated weekly. Serum biochemicals concentrations (glucose, cholesterol, triglycerides, total protein, cortisol and insulin) were estimated on the 42th, 72 th, 102th, and 132th, day. The obtained results indicated that dietary Chromium Methionine supplementation produced significant increase in milk production and 7% F C M during the entire period ($P < 0.05$), but no significant change on milk composition. Supplemental Chromium had no significant effect on serum glucose, cholesterol, triglycerides and insulin concentrations. On the other hand, significant increase in total serum protein and decrease in cortisol levels ($P < 0.05$) were recorded.

Key words: buffaloe cows, Chromium Methionine, milk yield, serum biochemistry

Introduction

Chromium has been reported to play essential roles in activity of certain enzymes, metabolism of protein nudeic acids as well as impact on immune functions [4]. Chromium aids in conversion of thyroxin to triiodothyronine and increasing metabolic rate [6]. Potential benefits of supplementating chromium to livestock have been shown to improve performance in growing and finishing ruminants [9, 12]. The element has been shown to increase dry matter intake and milk yield [8, 15]. Since there is no adequate measure of Chromium status, thus establishing dietary requirement for livestock and human remain a problem. While the recommended intake for chromium is 50 – 200 μg per day [13] in human. Currently there is no established Chromium requirement for ruminants.

The aim of the current study was to asses the effect of supplemental Chromium for lactating buffaloe cows, from 6 WKs to 19 WKs postpartum on performance and blood serum biochemistry.

Material and methods

Our wrok was conducted on thirty lactating buffaloe cows (3 – 6 years old and aver. B. Wt. 567 ± 22.78 kg) in the 3rd and 4th lactation period. Animals were belonged to experimental farm at Faculty of Agriculture, South Valley University. The work was done from October 2011 to February 2012.

Experimental animals and rations

Animals at peak of their lactation curve (From 6 – 19 WKs) were equally divided into two groups. Control group (G1) feeds on control ration consisted of concentrated feed mixture (CFM) and rice straw (RS) and treated group (G2) feeds on the control ration to which Chromium Methionine (Cr. Met. 5 mg/head/day), via ball dough corn was given after the a. m milking from 6 – 19 WKs after parturition.

Feed samples: The concentrated feed mixture (CFM) contains 33% yellow corn, 33% wheat bran, 20% rice bran, 11% decorticated cottonseed meal, 2% calcium carbonate and 1% sod. Chloride and rice straw (RS) were analyzed [1]. Mineralized salts licking blocks were available throughout the experiment. Animals had ad libitum access to clean water 24 hrs., a day. Animals were adapted to the double daily mechanical milking at 5.0 a. m and 4.0 p. m.

Table 1 The proximate analysis of concentrate feed mixture (CFM) and rice straw (Rs)

Nutrients (%)	Feeds		Nutrients (%)	Feeds	
	CFM	RS		CFM	RS
D M	99.40	93.40			
Ch. Comp. (% as MD)					
OM	88.39	85.39	EE	2.20	1.80
CP	15.60	4.40	CF	11.35	41.24
			NFE	59.24	37.89
			Ash	11.61	14.67

Milk samples: Milk yield was recorded individually and daily, twice a day for each animal. Individual milk samples consisted of proportional valumes of morning and evening milk were collected for analysis [1]. Solid non fat (SNF) was calculated by the difference (T. S% - Fat%). Milk yield was corrected to 7% fat for animals [14].

Blood samples: Blood samples were collected on the 42th, 72th, 102th, 132th day of lactation from each animal. Blood Serum was stored at −20℃ for a maximum of 60 days until assayed. Glucose, total cholesterol, triglycerides and total protein of serum were measured [7]. Insulin and cortisol were determined [2, 18].

Statistical analysis: The obtained data were statistically analyzed [17].

Results and discussion

Milk yield and composition

As shown in Table 2, Cr. Supplementation had significantly increased milk yield and fat milk concentration (7% FCM) that agreed with [3, 8, 16]. The element did not significantly affect the chemical composition of milk that agreed with [3, 15]. On the other hand, increased fat content in milk after Cr. Supplementation was reported [8].

Table 2 Effect of feeding buffaloe cows on chromium supplemented ration on milk yield and its composition

Items	G1 (Control)	G2 (Treated)	Items	G1 (Control)	G2 (Treated)
No.	15	15	No.	15	15
Milk yield (kg/d)	8.05 ± 0.11[b]	8.75 ± 0.17[a]	SNF %	9.71 ± 0.07	9.73 ± 0.10
7% FCM (kg/d)	10.19 ± 0.23[b]	10.65 ± 0.21[a]	T. Solid %	17.39 ± 0.56	17.67 ± 0.45
Fa + %	7.68 ± 0.43	7.94 + 0.44	Ash %	0.78 ± 0.04	0.77 ± 0.03
Protein %	4.22 ± 0.03	4.23 ± 0.03			

[a-b] Means in the same row followed by different letters are significantly different (P < 0.05).

Table 3 Effect of feeding rations on some blood parameters

Items	G1 (Control)	G2 (Treated)	Items	G1 (Control)	G2 (Treated)
No.	15	15	No.	15	15
Glucose (mg/dl)	52.58 ± 1.7	51.17 ± 4.11	T. protein (mg/dl)	7.02 ± 0.18[b]	7.39 ± 0.18[a]
T. cholesterol (mg/dl)	146.5 ± 14.2	143.2 ± 14.23	Insulin (μl/ml)	16.03 ± 3.22	16.5 ± 3.17
Triglycerides (mg/dl)	48.91 ± 4.4	52.83 ± 4.37	Cortisol (μl/ml)	38.4 ± 5.95[a]	26.2 ± 5.56[b]

[a-b] Means in the same row followed by different letters are significantly different (P < 0.05).

Blood serum biochemical parameters

Blood serum glucose and insulin concentrations had not been affected by Cr. supplementation (Table 3), that were similar to findings of [3, 5] on cows and [11] on sheep. On the contrary, [6] on Cows and [10] on Calves studies had reported that Cr. Supplementation caused decrease in glucose concentration and increase of insulin. Cr. Supplementation significantly (P < 0.05) decrease serum cortisol (Table 3), that was similar to the findings of [12] in calves, which might be due to nutritional stress. Blood serum cholesterol and triglycerides were not affected, that can be explained on basis of disability of chromium on insulin and fat metabolism [10]. Significant (P < 0.05) elevation in total serum protein (Table 3) was similar to [6], that could be due to insulin concentration and increased sensitivity of tissues to it.

Conclusion

It could be concluded that Cr. Supplementation significantly increased milk yield but not its composition during the entire period. Biochemical findings revealed elevation in total serum protein, decrease in serum cortisol and no change in the other studied constituents.

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Effect of *Aspergillus niger* and *Trichoderma longibrachatium* in Degradability of Dry Matter and NDF from Alfalfa

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Summary: We evaluated *in situ* a xylanase enzyme site (Fibrozyme, Alltech, Inc.) consisting of fermentation extracts and *Aspergillus niger Trichoderma longibrachatium* at different ruminal incubation periods (48, 24, 12, 8, 6, 4 and 2 h) alfalfa hay. The variables were: DMD and NDFD and nonlinear parameters. T2 (adding enzyme) was higher ($P < 0.05$) in DMD and NDFD per period and completely, obtaining an effective degradability and passage rate higher ($P < 0.01$) than T1. The addition of the xylanase enzyme rumen accelerated in the DMD in a shorter time (6 – 18 h) and NDFD (2 – 12 h). We conclude that adding the xylanase enzyme improves in less time degradability of DM and NDF, expecting increased feed intake and animal production.

Introduction

Exogenous enzymes in feed for ruminants affect fiber digestibility, DM and NDF (Tricarico et al., 1998). Fibrozyme (exogenous enzyme) positive effect ($P < 0.05$) in vitro DM degradability at 12 h in 44% (Tricarico et al., 1998), Guerra et al. (2003) did not differ ($P > 0.05$) this parameter in diets based on alfalfa more Fibrozyme site. Diets with 78% concentrate increased ruminal digestion of NDF (25%) (Zinn and Salinas, 1999), 18% in diets with 35% forage (Ambrozio et al., 2000), and 15.13% in diets with hay alfalfa (Guerra et al., 2003). The objective was to evaluate in situ the effect of the enzyme in these parameters Fibrozyme different ruminal incubation periods based rations of alfalfa hay.

Material and methods

Xylanase enzyme was evaluated (Fibrozyme, Alltech, Inc.) consisting of fermentation extracts and Aspergillus niger Trichoderma longibrachatium in two treatments T1 = alfalfa, alfalfa more Fibrozyme = T2. Basal diet: alfalfa hay *ad libitum* and 3 kg of concentrate d^{-1} (Fodder El Barrio), for ten days. The enzyme was dosed: 14 $g \cdot d^{-1}$, 7 ga 7 ga 0800 and 2000 h, two male animals holstein-gyr 3/4 cannulated in rumen weight of 800 kg. Introduced per animal per treatment 112 nylon bags 10 × 20 cm (ANKOM) with 5 g of ground sample (1 mm) of alfalfa, weighed and identified by bag, animal and period, with a pore size of 50 – 15 μm, and exhibition area of 18 $cm^2 \cdot mg^{-1}$. DM and NDF was estimated (AOAC, 1975). The degradability of dry matter (DMD) and degradability of neutral detergent fiber (NDFD) was evaluated at different incubation periods 48, 24, 12, 8, 6, 4 and 2 h. Subsequently the bags water jet washed for 5 min, to be clean. For in situ degradability formula was used Schneider and Flatt (1975). Nonlinear parameters were estimated rumen (NLPR) of DMD and NDFD (Ørskov and McDonald, 1979) with the exponential equation: $p = a + b\ (e^{-ct})$, where: p = rate of disappearance of nutrients in a while, a = intercept of the solubilized portion at the beginning of the incubation (time 0), b = potentially degradable fraction in the rumen, c = speed or rate of degradability of fraction b, and t = time of incubation. Potential degradability was obtained (pd) = a + by effective degradability (ed) = $a + b * c/(c + 0003)$ assuming a passage rate of 3% (Ørskov and McDonald, 1979). DBCA was employed, proof χ^2 and Tukey mean comparison (Barreras et al., 1997). Statistical analyzes were performed using the SAS statistical package (2001).

Results and discussion

The DMD was different ($P < 0.05$) for treatment periods. T2 showed superiority from 6 to 18 h, more efficiently: between 16.53% – 15.66% (Fig. 1). The total DMD undifferentiated periods shows that T2 exceeded ($P < 0.05$) to 12.31% in T1 (T1 = 56.68^{b}% *vs.* 63.66^{a}% = T2).

The NLPR for DMD were different ($P < 0.01$) for all components, highlighting (Table 1) T2 who less potential degradability (73.257^{b}%) T1 (73.994^{a}%)

showed a higher effective degradability ($P < 0.01$) of 66.670^{a} *vs.* 64.550^{b} by greater ($P < 0.01$) degradability rate (18.49^{a} *vs.* 11.98^{b}% h^{-1}).

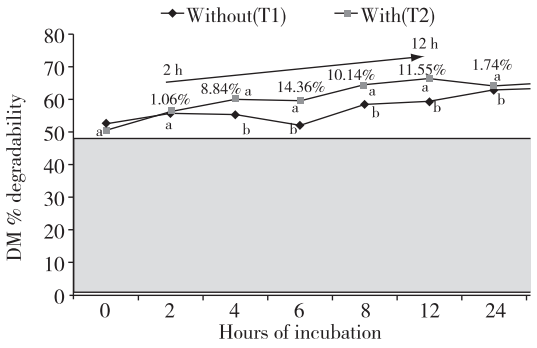

Fig. 1 DM% degradability
Literal values differ statistically different ($P < 0.05$) Tukey test. (C. V. = 2.21 – 18.0)

Table 1 Nonlinear parameters *In situ* ruminal degradability for DM and NDF

Parameters	DM	
	T1 (without)	T2 (with)
a	26.844^{a}	26.107^{b}
b	47.150^{a}	47.150^{a}
c (h – 1)	0.1198^{b}	0.1849^{a}
pd (a + b)	73.994^{a}	73.257^{b}
ed	64.550^{b}	66.670^{a}
Parameters	**NDF**	
	T1 (without)	T2 (with)
a	52.596^{a}	50.483^{b}
b	12.250^{b}	14.858^{a}
c (h – 1)	0.0588^{b}	0.2419^{a}
pd (a + b)	64.846^{b}	65.341^{a}
ed	60.707^{b}	63.703^{a}

Values in the same row with different literal differ statistically ($P < 0.01$) X2.

The same trend was presented to the NDFD, differing ($P < 0.05$) treatment periods $^{-1}$. T2 after 2 h at 12 claims to be more efficient ($P < 0.05$) in the range of 1.06 to 14.36% (Fig. 2), and a 7.11% in the overall mean comparison (*vs.* T1 = 58.16^{b}. 62.30^{a}% = T2). The NDFD NLPR of evidence ($P < 0.01$) effective degradability 63.703^{a} *vs.* 60.707^{b} and degradability rate 24.19^{a} h^{-1} (T2) and 5.88^{b} (T1) (Table 1). This shows that using exogenous enzymes in feed for ruminants increase the use of DM and NDF (Tricarico et al., 1998, Guerra et al., 2003).

Conclusions

We conclude that adding DMD Fibrozyme improved in less time (6 – 8 h) and NDFD (2 – 12 h), expecting increased feed intake and animal production.

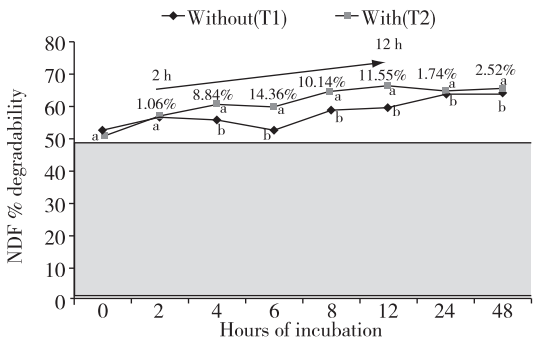

Fig. 2 NDF h^{-1} degradability
Literal values differ statistically different ($P < 0.05$) Tukey test. (C. V. = 0.83 – 8.67).

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Effects of High Dietary Zinc and Biotin Levels on Development and Severity of Foot Pad Dermatitis in Broilers Housed on Litter with Critical Moisture Content

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Summary: This experiment was designed to test the hypothesis that higher levels of zinc (methionine or oxide) and biotin than are normally present in commercial diets can reduce/prevent foot pad dermatitis (FPD) in broilers reared on litter with critical moisture content (35% water). Two trials were done, in each four groups of 1-wk-old ♂ broilers were reared during 33 days. All groups (25 birds in each) were littered with wood shavings (5 kg/m^2) of critical moisture content (35% water) experimentally maintained by adding water. Two groups were fed upper levels of Zn as zinc-oxide (150 mg Zn/kg diet) with control level of biotin (300 μg/kg diet) or high biotin (2000 μg/kg diet). The other two groups were fed Zn as zinc-methionine (150 mg Zn/kg diet) with control level of biotin (300 μg/kg diet) or high biotin (2000 μg/kg diet). External assessment of foot pads and weighing of birds were done weekly. Moisture contents of litter and excreta were measured weekly and at trials' end, too. The results showed that high biotin supplementation was accompanied with a reduction by ~ 40% and 20% (trials 1/2) of foot pad lesions. Using dietary Zn-Met in comparison to ZnO showed a decrease in the severity of FPD lesions by (~10%) in both trials. The combination of Zn-Met and high biotin levels led to reduced scores at poor litter conditions by ~50% (trial 1) and 30% (trial 2). No significant differences were found among the experimental groups for the performance parameters; however, high dietary biotin levels were accompanied with the highest final body weight. It is recommended to combine the upper level of zinc (especially of Zn-Met) and high levels of biotin (~2000 vs. normal levels of ~300 μg/kg) when clinically relevant alterations in foot pad occur.

Introduction

For many decades foot pad dermatitis (FPD) has been known as a wide spread problem in poultry production. Recently it has attracted additional attention in terms of animal welfare, food safety and also consumer protection. FPD is a type of contact dermatitis with hyperkeratosis in its early stage followed by necrosis and ulcers of the foot pads in its late stage [5]. The aetiology of FPD is a complex interaction of different factors [8]. One common aspect in most previous studies is that litter moisture is the predominant factor in the onset of FPD. The first marked increase in FPD lesions in turkey poults occured after exposure for only 4 h/day to a critical litter moisture' of 35% [1].

Buda [3] observed that a high level of biotin (~2000 μg/kg diet) had a positive effect on FPD, whereas MAYNE et al. [9] did not found any effect of a high dietary biotin level (1600 μg/kg diet) whatsoever. Moreover, zinc deficiency causes dermatitis and immunological dysfunction [11]. Since Zn supplementation was found to be essential, the NRC [10] recommended 40 ppm for broiler chickens, which appeared to be based on results which considered growth performance as the only criterion. In avian species, methionine is classified as a first limiting amino acid because of it being limited in plant protein sources and the strong requirement for it to support feather growth and protein synthesis. Deficiency in methionine consumption has a significant negative impact on animals such as growth inhibition, the induction of metabolic disorder and the reduction of disease defensive potential [4]. The main objective of this study was to test the hypothesis that higher concentrations of biotin and zinc (methionine or oxide) than normally present in commercial diets (supposed to meet animals' requirements) might have preventive effects regarding the development and severity of FPD in broilers exposed to conditions resulting in high alterations of the foot pad.

Material and methods

Two experimental trials were performed. In each trial, 100 birds having normal/health foot pads were chosen randomly for the beginning of the experiment (d 8). The birds were leg-banded individually and then divided into 4 equal groups, each housed in a floor pen (1.50 m × 1.32 m). All groups were kept on wood shavings with a moisture content of 35% (as a challenge). The wet litter was experimentally maintained by adding water as required (day after day). The added amount of water was estimated in pre-experimental

studies, and then modified during this experiment by measuring the dry matter (DM) content of litter continuously. In each group, the depth of the litter material was approximately 4 cm (5 kg/m^2 of wood shavings). The four experimental diets were fed to the birds at the beginning of the second week (d 8) up to the end of the fattening period (d 40). Two groups were fed high levels of Zn (150 mg/kg diet) as zinc-oxide (ZnO), with normal levels of biotin (300 μg/kg diet) or high biotin levels (2000 μg/kg diet). The other two groups were also fed high Zn (150 mg/kg diet) as zinc-methionine (Zn-Met) with normal levels of biotin (300 μg/kg diet) or high biotin levels (2000 μg/kg diet). A biotin powder and Zn-Met were used to supplement the high biotin diets and the organic source of Zn. The composition and chemical analysis of the different experimental diets are presented in Tables 1 and 2. The vitamin and mineral mixtures were commercially produced so as to be free from biotin and zinc. Litter samples for measuring moisture and pH were collected weekly. Individual body weight was recorded weekly on the day of scoring. Feed and water intakes were measured daily at group level. Feed conversion ratio (FCR) was estimated and corrected for mortalities (4 of 200 birds). External assessment of foot pads was done weekly. During the external examination, if the feet were dirty, they were gently washed with a wet cloth and dried before scoring; only the central plantar area was scored, signs of foot pad lesions were recorded on a 7-point scale according to MAYNE et al. [9].

Results

Wet litter was experimentally maintained in all groups by adding water as required to achieve approximately 35% moisture. Thus, no marked differences were found in the mean of moisture content of the litter among the experimental treatments. Table 1 shows that no significant differences among the experimental groups at d 40 in the first trial were observed. However, birds fed high levels of biotin combined with Zn-Met showed numerically the highest body weight (2219 g) compared with the other groups. Also, in the second trial no significant differences were observed among the experimental groups. Nonetheless, birds fed high levels of biotin combined with ZnO showed numerically the highest body weight (2511 g) compared with the other groups (Table 1). In both trials, the lowest final body weight was found in birds fed the normal level of biotin combined with Zn-Met (2012 and 2405 g in trials 1 and 2, respectively). Moreover, feeding the normal level of biotin combined with Zn-Met was accompanied by the highest FCR in both trials (1.57 and 1.67). A favourable FCR (Table 1) was observed for birds fed the normal level of biotin with ZnO (1.51 and 1.63 in trials 1 and 2, respectively).

From a statistical point of view (two factors analysis

Table 1 Comparison of broiler's body weight (g) and FCR (Submitted to Poultry Science)

	Level of biotin (μg/kg)	Source of zinc	Day (duration of treatments) 14 (7) (n=25)	21 (14) (n=25)	28 (21) (n=25)	35 (28) (n=25)	40 (33) (n=25)	Corrected FCR
trial 1	300	oxide	267a ±36.2	577^{a} ±93.2	982^{a} ±167	1555^{a} ±253	2145^{a} ±360	1.51
	2000		280^{a} ±28.5	592^{a} ±71.7$^{1)}$	996^{a} ±145$^{1)}$	1569^{a} ±232$^{1)}$	2179a ±313$^{1)}$	1.54
	300	methionine	274^{a} ±39.5	569^{a} ±86.1	965^{a} ±157	1521^{a} ±248	2012^{a} ±317	1.57
	2000		270^{a} ±41.1	580^{a} ±108$^{1)}$	971^{a} ±183$^{1)}$	1570^{a} ±293$^{2)}$	2219^{a} ±363$^{2)}$	1.51
trial 2	300	oxide	433^{a} ±35.6	794^{ab} ±85.1	1307^{a} ±178	1962^{a} ±285	2468^{a} ±348	1.63
	2000		449^{a} ±27.0	822^{a} ±66.6	1350^{a} ±145	2012^{a} ±246	2511^{a} ±309	1.63
	300	methionine	428^{a} ±38.2	755^{b} ±101$^{1)}$	1262^{a} ±204$^{1)}$	1912^{a} ±320$^{1)}$	2405^{a} ±399$^{1)}$	1.67
	2000		433^{a} ±39.2	774^{ab} ±88.7	1309^{a} ±155	1952^{a} ±257	2431^{a} ±298	1.65

^{a,b}Means in the same column with different superscripts in each trial are significantly different ($P<0.05$).

Table 2 External scores of foot pads in broilers fed different experimental diets (two factors variance analyses; Mean ± SD) Submitted to Poultry Science

	Factor	Treatment	Day (duration of treatments)/FPD scores 14 (7) n=50	21 (14) n=49	28 (21) n=49	35 (28) n=49	40 (33) n=49
tria 1	biotin	control	0.7^{a} ±0.4	1.0^{a} ±0.3$^{1)}$	1.9^{a} ±0.9$^{1)}$	3.6^{a} ±0.9$^{1)}$	4.8^{a} ±1.0$^{1)}$
		surplus	0.6^{a} ±0.4	0.9^{b} ±0.3$^{2)}$	1.2^{b} ±0.4$^{2)}$	1.6^{b} ±0.7$^{3)}$	2.7^{b} ±1.1$^{3)}$
	zink	oxide	0.7^{a} ±0.4	1.1^{a} ±0.3	1.9^{a} ±0.9	2.9^{a} ±1.3	4.1^{a} ±1.2
		methionine	0.5^{b} ±0.5	0.9^{b} ± 0.2	1.3^{b} ± 0.5	2.4^{b} ±1.3$^{2)}$	3.6^{a} ±1.7$^{2)}$
tria 1	biotin	control	0.8^{a} ±0.4	1.3^{a} ±0.7	2.1^{a} ±1.2	3.4^{a} ±1.3	4.4^{a} ±1.0
		surplus	0.5^{b} ±0.4	1.1^{a} ±0.4	1.8^{a} ±0.8	2.7^{b} ±0.9	3.6^{b} ±1.0
	zink	oxide	0.7^{a} ±0.4	1.3^{a} ±0.6	2.2^{a} ±1.1	3.3^{a} ±1.2	4.2^{a} ±1.0
		methionine	0.6^{a} ±0.4	1.1^{a} ± 0.5	1.7^{b} ± 0.9	2.8^{b} ±1.0	3.7^{b} ±1.0

^{a,b}Means in the same column in each factor with different superscripts are significantly different ($P<0.05$).
$^{1)}$ n=50, $^{2)}$ n=48, $^{3)}$ n=47

of variance) Table 2 shows that in both trials feeding surplus levels of biotin resulted in significantly decreased FPD scores (2.7 ± 1.1 and 3.6 ± 1.0 in trials 1 and 2, respectively) in comparison to groups fed normal levels of biotin (4.8 ± 1.0 and 4.4 ± 1.0 in trials 1 and 2, respectively). Furthermore, in both trials (Table 2) feeding ZnO diets were accompanied by markedly higher FPD scores (4.1 ± 1.2 for first trial and 4.2 ± 1.0 for second trial) compared with groups fed Zn-Met diets (3.6 ± 1.7 and 3.7 ± 1.0 in trials 1 and 2, respectively).

Discussion

Moisture is the key factor influencing litter quality and managing litter is a crucial step in promoting flock health and well-being [8]. The dietary treatments did not markedly affect the DM content of the litter. The DM content in all pens was maintained at 35% by adding water as required. However, the addition of water was stopped altogether in all pens at the second week of the experiment, when the moisture content of litter reached 35%.

Body weight was influenced by biotin supplementation (in each Zn source) for both trials. This finding could be due to biotin being required in carbohydrate metabolism, fatty acids, protein synthesis and nucleic acid metabolism formation [12]. Contrary to these findings, HARMS and SIMPSON [6] stated that the body weight of turkey poults was not influenced by biotin supplementation (220 μg/kg diet).

The present results suggest that increasing dietary biotin supplementation above normal recommendations for broilers will reduce the severity of FPD in spite of continuous exposure to wet litter. Some more up-to-date experiments have demonstrated that biotin supplementation is still required to minimise FPD as commercial diets appear not to contain adequate levels of biotin to prevent FPD. Our results support the findings of Buda [3] that very high concentrations of biotin (2000 μg/kg diet) result in a reduction in the severity of foot pad lesions in comparison to commercial diets containing an average of 300 μg/kg in turkey diets. YOUSSEF et al. [13] mentioned that a high concentration of biotin (2000 μg/kg diet; 47 mg Zn/kg diet) reduced the development and severity of FPD on dry litter (25% moisture), but not on very wet litter (73% moisture). Also, MAYNE et al. [9] reported that high dietary concentrations of biotin (1600 μg/kg) did prevent the occurrence of FPD in growing turkeys reared on wet litter (45% moisture. With regard to the effect of organic zinc, the severity of FPD was marked lower in birds fed Zn-Met in comparison to those fed ZnO diets. HESS et al. [7] noted that FPD lesions were reduced significantly in female broilers fed diets containing Zn-amino acids complexes. Similarly, BILGILI [2] reported that dietary zinc from organic sources decreased the incidence and severity of FPD even under conditions of high stocking density.

Conclusions

The present results indicate that it is recommended to combine the maximum levels of zinc (especially of Zn-Met) and high levels of biotin (~2000 vs. normal levels of ~300 μg/kg) when clinically relevant alterations in foot pads occur (additive positive effects). However, the supplementation of these additives in the diet depends on their price, Zn being cheaper than biotin. The tested dietary content of biotin (2000 μg/kg diet) can be used in unlimited amounts as it has no toxic or environmental effects.

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Role of Anti-oxidants in Reducing Aflatoxicosis in Chickens

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Summary: Mycotoxicoses are diseases caused by consumption of diets contaminated with mycotoxins, a special class of fungal secondary metabolites. Aflatoxin B_1 (AFB_1) and Fumonisin B_1 (FB_1) are the main toxins synthesized by toxicogenic stocks of *Aspergillus* spp. and *Fusarium* spp. respectively and can coexist in grains and in its by-products. The other types of mycotoxins are ochratoxins, patulin, trichothecenes and zearalenone). they all represent the main problems for human and animal health in the world.

Aflatoxins can enter the food chain through contaminated cereals and foods (e.g., milk, meat, and eggs) obtained from animals fed aflatoxin-contaminated feeds. Aflatoxins (AF) have a high impact in both human and animal health, causing significant economic losses in the poultry industry, especially by diminution of avian growth, feed efficiency, and product quality. Aflatoxins affect the whole organism, particularly liver and kidney.

Mycotoxins chemical, biological, and toxicological properties are diverse. Hence, their toxic effects are extremely variable, depending on the intake level, duration of exposure, animal species, age, sex, physiological status, and eventual synergism between mycotoxins simultaneously present in feed or foods. However, the main toxic effects for aflatoxicosis, oxidative stress is a common mechanism contributing to initiation and progression of hepatic damage. When animals or humans consume these foods, AFB_1 is metabolized in the liver producing the formation of highly reactive chemical intermediaries.

Because lipid peroxidation plays a major role in the toxicity of AF, a protective effect of antioxidants is possible. Antioxidants like vitamins (E, A and C), carotenoids, selenium and other antioxidants material have inhibitory action on biotransformation of AF to their active epoxide derivatives, and have beneficial effects in ameliorating the adverse effects resulting from AF.

Introduction

Aflatoxins are secondary metabolites produced by *Aspergillus flavus* and *A. parasiticus*, and they have carcinogenic, mutagenic, hepatotoxic and teratogenic effects [16]. Aflatoxins contaminate food and feed worldwide, causing several diseases are associated with the human consumption of these toxins, including toxic hepatitis and even primary hepatocellular carcinomas [19]. Furthermore, a wide spectrum of toxic responses is related to the exposure of animals to aflatoxins, with most of them causing economical losses resulting from decreased production. AFB1-mediated toxicity was also found to be related to its pro-oxidant potential. This is because reactive oxygen species (ROS) including superoxide anion (O^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (-OH) are generated during the metabolic processing of AFB1 by liver enzymes [21,26]. ROS cause oxidative stress by damaging cellular membranes and components. Therefore, it can be assumed that natural components having antioxidant potential are capable of inhibiting ABF1-induced oxidative damage either by scavenging ROS or stimulating antioxidant defense systems [35]. Animals of different species vary in their susceptibility to acute aflatoxin poisoning with LD_{50} values ranging from 0.3 to 17.9 mg/kg b. wt. (Table 1). Factors that influence aflatoxin-toxicity residue levels in animal species include: species and breeds of animals and poultry, levels and duration of exposure, nutrition and health of animals, age, sex and diseases, drugs and other mycotoxins[9,10].

Detoxification of aflatoxin:

Detoxification of aflatoxin contaminated foods and feeds is a current problem, as aflatoxins are highly carcinogenic and capable of passing unaltered through metabolic processes and accumulating in the tissues (seriously jeopardizing human and animal health). Although numerous detoxification methods have been tested, none seems able to fulfill the efficacy, safety, safeguarding of nutritional elements and costs requisites of a detoxification process [20]. For that, studies have never stopped to evaluate the efficacy of the available detoxification methods and to develop recent innovations in this field to reduce the bioavailability of aflatoxin to poultry and farm animals.

Table 1 A comparison of single oral LD_{50} values for AFB1 in various species

Toxin	Animal	Age/size	LD_{50} (mg/kg)	Reference
AFB1			0.37	[32]
AFB2			1.69(84.8 μg/50 mg duckling)	[31]
AFG1	Duckling	Day-old	0.79	[15]
AFG2			2.5(172.5 μg/duckling)	[2,15]
AFM1			0.8(16.6 μg/duckling)	[2]
	Rabbit		0.3 – 0.5	[17]
AFB1	Turkey	Young foals	0.5 – 1.0	[32,33]
	Chickens		6.5 – 16.5	[23]

The range of aflatoxin that can contaminate poultry feed and their different chemical composition make protection against mycotoxin related toxicity a difficult task. There are various approaches to control or combat mycotoxin problems. The simplest strategy is based on the prevention of the formation of mycotoxin in feeds by special management programmers including storage at low moisture levels and prevention of grain damage during processing[6].

Other strategies based on the use of microbial or thermal inactivation of toxins, physical separation of contaminated feedstuffs, irradiation, ammoniation and ozone degradation have not developed to the industrial level because they are either time consuming or comparatively expensive[7]. In recent years, nutritional manipulation has been actively detrimental consequences of mycotoxin consumption.

Any decontamination process must be technically and economically feasible if it is to be applied practically. The FAO requirements for acceptable decontamination process [9] stipulate that the procedure must:

1. destroy, inactivate or remove aflatoxins; 2. not produce nor leave toxic and/or carcinogenic/mutagenic residues in the final products or in food products obtained from animals fed on decontaminated feeds; 3. not significantly alter the important technologic properties; and ideally; 4. destroy fungal spores and mycelium that could proliferate and produce new toxins under favorable conditions. Analogous requirements have been set down in France and U. S. A., however the F. D. A. [5] requires additional data on the impact of the process to the environment.

Protective effect of antioxidants against aflatoxin:

Since lipid peroxidation plays an important role in mycotoxin toxicity, a protective effect of antioxidants is expected [10]. Indeed, several experiments with various animal species, protective effect of antioxidants against toxic effect of mycotoxine were observed. For examples, vitamin E supplementation ameliorated the pro-oxidative effect of aflatoxin[27].

Other antioxidant compound also has protective effect against various mycotoxin. Selenium (Se) is shown to have protective effects against aflatoxin. The experimental results provided clear evidence of Se induced enhancement of aflatoxin detoxification [12]. It was suggested that the protective action of Se was not mediated by an increase in glutathione availability for aflatoxin conjugation or by effect on activities of these enzymes as measured *in vitro*. more over a synthetic seleno-organic compound showed a potent protective effect against AFB1 induced cytotoxicity[34]. It has been shown that Se can inhibit the formation of hyperplastic foci and enzyme altered foci as well as hepatocarcinogenesis induced by AFB1, but Se can neither prevent the enlargement nor accelerate the regression of the foci already developed after administration of carcinogens [29]. Therefore [14] concluded that Se had an inhibitory effect on the initiation and promotion stages of AFB1 induced preneoplastic foci and nodules. Selenium also prevented progression of these nodules to hepatocellular carcinoma even after cessation of AFB1 administration.

Ascorbic acid is also effective against mycotoxin [24]. It is known that carotenoids are also effective antioxidants *in vivo* and *in vitro* [25]. In this respect, lycopene [13] and canthaxanthin has some properties against aflatoxicosis [28] Dietary carotenoids inhibited AFB1 induce liver DNA damage [11] and xanthophylls inhibited the mutagenicity of AFB1 in a dose dependent manner [8]. The author suggested that the inhibitory mechanism of lutein against AFB1 mutagenicity is the result of formation of a complex between lutein and AFB1, direct interaction between lutein and AFB1 metabolism and modulation of the metabolic activation of AFB1 by lutein.

Vitamin A also had reduced AFB1 induced DNA damage[30]. AFB1 induced hemolysis was significantly reduce on adding vitamin A in incubation medium [18]. Two vitamin A compounds (3-dehydroretinol and 3-dehydroretinyl palmitate) have been found to inhibit the microsome catalyzed formation of DNA adduct by AFB1 [1].

Synthetic antioxidants can also be effective against

mycotoxicoses. For example, addition of liver cytosolic fractions prepared from the rat pretreated with high dose of BHT to the cell free system caused a 48% inhibition in AFB1-DNA binding[24].

Antioxidants enzymes (SOD and catalase), when applied to Vero cell *in vivo* prior to OA, prevented lipid peroxidation [4] as well as most of the neutrotoxic effects induced by OA [3]. Similarly, in cultured rat hepatocytes SOD and catalase protected against AFB1 induced MDA formation and cell injury [22].

Conclusions

- Mycotoxin in feed (at least OA, T-2 toxin and AFB1) cause malabsorption in the intestine, which results in impaired absorption and decreased concentration of vitamin E, C, and carotenoids in tissues. Mycotoxins promote free radical formation (O_2^- and OH^*) in the intestine, which cause antioxidant depletion, oxidative stress, enterocyte apoptosis and contribute to the development of malabsorption and decreased antioxidant absorption and accumulation.
- Mycotoxins and their active metabolites are absorbed from the intestine and accumulated in target tissues.
- Mycotoxins in tissues can generate free radicals, decreasing future antioxidant protection, causing lipid peroxidation and damage to other biological molecules including lipids, proteins and DNA. This could lead to antioxidant/pro-oxidant imbalance causing oxidative stress, which further leads to apoptosis and other cytotoxic effect of mycotoxins.
- Increased antioxidant supplementation protects against toxic actions of mycotoxins by interfering with one or several steps described above, including gastrointestinal tract, plasma and tissue membranes.
- A combination of natural antioxidants with mycotoxin binders could be a next step in combating mycotoxicoses in poultry production.

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Effect of Feed Additives Containing *K. daigremontiana* and *M. sativa* L. on Egg Traits and Performance of Laying Hens

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Summary: The aim of the study was to evaluate the effect of feed additives containing *Kalanchoe daigremontiana* and *Medicago sativa* L. on the performance and egg traits of laying hens. A total of 150 laying hens (Hy-Line Brown), 25 week of age, was divided into 6 groups (5 replicates), and a feeding experiment was conducted for 5 weeks. The basal diet, was supplemented with the pulp of *Kalanchoe daigremontiana* leaves or the phyto-mineral feed preparation (vermiculite, bentonite, calcium, humodetrynite form of brown coal) containing the pulp of *Kalanchoe d.* leaves or containing the pulp and *M. sativa* L., at the doses of 5.0% wt. The investigated feed additives did not significantly affect feed intake (0.122 – 0.127 kg/hen-d) or hen-day egg production (94.29% – 98.63%). Moreover, Haugh units (94.56 – 99.02), shell thickness (0.351 – 0.368 mm) and eggshell strength (3.55 – 4.00 kg) were not influenced by examined diets. However, the phyto-mineral feed preparation affected the color of the yolk and the results estimated with a La Roche color fan were confirmed with the use of Chroma meter. Thus, the results show no effect of examined feed additives on performance and the most of egg traits, but it cannot be excluded that positive effects would have been observed in long-term experiment or under less hygienic environmental conditions.

Introduction

Plant-derived products, used in animal feeding, recently gained increasing interest due to the fact that European Union banned most of the antibiotic additives. Therefore, an application of new feed additives, e. g. spices, herbs, essential oil blends or plant extracts for phytopreparations and phytobiotics production is increasing very fast [2, 6, 11]. Biologically active substances contained in natural raw materials demonstrate multidirectional activity i. e. antibacterial, antifungal, reducing an oxidative stress in animals or exhibit preventive and therapeutic activity [3, 7]. *Kalanchoe* plants contain components of bactericidal and fungicidal properties and demonstrate regenerative, anti-inflammatory and immunostimulating properties. Plants of the *Kalanchoe* genus also found an application in veterinary [1]. Moreover, bufadienolides isolated from the leaves of *Kalanchoe* were found to be potential cancer chemopreventive agents [10]. The aim of the study was to evaluate the effect of feed additives containing *Kalanchoe daigremontiana* and *Medicago sativa* L. on the performance and egg traits of laying hens.

Material and methods

A total of 150 laying hens (Hy-Line Brown), 25 week of age, was divided into 6 groups (5 replicates), and a feeding experiment was conducted for 5 weeks. The investigation was conducted in a room with controlled climate and light regimen of 16L: 8D. Hens were housed in a 3-tier battery system. Feed and water were provided *ad libitum*. The basal diet was formulated to be isocaloric and isonitrogenous according to nutrient recommendations for laying hens [9] and mainly composed of wheat (22.7% – 30.3%), soybean meal (22.7% – 24.5%), corn (20.0%) and barley (15.0%). The phyto-mineral feed preparation was manufactured on the basis of natural raw materials (vermiculite, bentonite, calcium and humodetrynite form of brown coal) using four-stage technology including hydro-thermal processes [8]. The pulp of *Kalanchoe daigremontiana* leaves was preserved with refined glycerine (99.7% purity, Bio-Chem, Poland) in 1: 1 ratio.

Hens were assigned to the one of the 6 following treatment groups:

- Control group (C) – standard feed mixture;
- Pulp group (Pulp) – standard feed mixture with addition of the pulp of *Kalanchoe d.* leaves, at the doses of 5.0% wt.;
- Phyto-mineral preparation 52 group (Pp52) – standard feed mixture with addition of the phyto-preparation containing 52.0% wt. of the pulp of *Kalanchoe d.* leaves, at the doses of 5.0% wt.;
- Phyto-mineral preparation 52α group (Pp52α) –

standard feed mixture with addition of the phyto-mineral preparation containing 52.0% wt. of the pulp of *Kalanchoe d.* leaves and 6.0% wt. of dried *Medicago sativa* L., at the doses of 5.0% wt.;

· Phyto-mineral preparation 36 group (Pp36) – standard feed mixture with addition of the phyto-preparation containing 36.0% wt. of the pulp of *Kalanchoe d.* leaves, at the doses of 5.0% wt.;

· Phyto-mineral preparation 36α group (Pp36α) – standard feed mixture with addition of the phyto-mineral preparation containing 36.0% wt. of the pulp of *Kalanchoe d.* leaves and 13.0% wt. of dried *Medicago sativa* L., at the doses of 5.0% wt.

Individual eggs were weighed as well as yolk, albumen and eggshells. Haugh units were calculated from the HU formula [HU = 100 log (H – 1.7W$^{0.37}$ + 7.57)]. Eggshell breaking strength was determined by using Zwick/Roell device. Eggshell thickness was measured on the egg equator at 2 points by a micrometer screw IP 54. Color of the yolk was measured using Chroma meter, model CR-400 (Minolta Corp.), and expressed according to the Hunter color values (L*, a*, b*). Additionally, yolk color intensity was visually estimated using a La Roche color fan.

Data were tested for normality (Shapiro-Wilk's test). Statistical comparisons between the groups were done via one-way ANOVA using Statistica, version 10.0 (Statistica for Windows, StatSoft Inc., Tulsa. OK). All the data are reported as means and effects were tested for significance using Tukey's multiple-range test, at a probability of $P < 0.05$ and $P < 0.01$.

Results and discussion

The effect of investigated feed additives on hen's performance and egg quality parameters is shown in table 1. Supplementation of the basal diet with the pulp of *Kalanchoe daigremontiana* leaves or the phyto-mineral feed preparations did not significantly affect feed intake and hen-day egg production. Moreover, the investigated egg quality parameters were not significantly different between the groups. However, the phyto-mineral feed preparation affected the color of the yolk and the results estimated with a La Roche color fan were confirmed with the Chroma meter.

Botsoglou et al. [4] were evaluating the effect of feeding rosemary, oregano, saffron and α-tocopheryl acetate on hen performance and egg quality parameters. Results showed no significant differences in egg production, feed intake, feed conversion ratio, egg weight and shape, yolk shape, Haugh units and shell thickness among treatments. However, yolk color was significantly improved in the saffron group compared to all

Table 1 Effect of investigated feed additives on performance and egg quality parameters (mean per egg, n = 5)

Item	Group						SEM	P value
	C	Pulp	Pp52	Pp52α	Pp36	Pp36α		
Performance								
Feed intake(g/hen/day)	0.122	0.123	0.122	0.123	0.124	0.127	0.0007	< 0.0001
Hen-day eggproduction(%)	95.09	96.69	95.20	94.29	98.63	95.89	0.5290	< 0.0001
Egg quality parameters								
Eggshell strength (kg)	3.85	4.00	3.90	3.55	3.88	3.83	0.0703	< 0.0001
Yolk color score1	5.25^{a}	4.60	4.16^{b}	4.11^{b}	4.08^{b}	4.63	0.1201	0.0720
Egg weight (g)	60.73	62.28	63.82	61.74	62.19	61.19	0.3873	< 0.0001
Haugh units	97.04	99.02	94.56	97.40	97.13	97.42	0.4622	< 0.0001
Egg albumen weight (g)	40.40	41.93	43.08	41.48	41.93	41.11	0.3471	< 0.0001
Egg yolk weight (g)	14.64	14.50	14.82	14.72	14.40	14.36	0.0996	< 0.0001
Eggshell weight (g)	5.69	5.84	5.93	5.54	5.86	5.72	0.0463	< 0.0001
Egg width (mm)	43.71	44.13	44.30	44.14	43.93	43.97	0.1088	< 0.0001
Egg height (mm)	56.22	56.76	57.49	56.31	56.79	56.20	0.1527	< 0.0001
Egg surface(cm^2)	73.27	74.49	75.66	74.05	74.39	73.64	0.3039	< 0.0001
Shape index	77.78	77.78	77.19	78.44	77.37	78.27	0.2243	< 0.0001
Shell thickness (mm)	0.37	0.36	0.36	0.35	0.36	0.36	0.0023	< 0.0001
L* value[2]	56.27^{Aa}	56.95^{a}	57.42	58.41^{b}	58.88^{B}	58.02	0.2307	< 0.0001
a* value[3]	–5.28^{a}	–6.82	–6.78	–6.92^{b}	–6.70	–6.29	0.1694	< 0.0001
b* value[4]	36.53	36.84	36.32	34.16^{A}	35.89	39.00^{B}	0.3885	< 0.0001

$^{A-E}$ values within the same row with different superscript letters differ($P < 0.01$);

$^{a-e}$ values within the same row with different superscript letters differ($P < 0.05$);

[1] Yolk color score estimated with a La Roche color fan;

[2] L* values, 0 = dark, 100 = bright;

[3] a* values, positive = red, negative = green;

[4] b* values, positive = yellow, negative = blue.

other groups. Moreover, authors were suggesting that hens may not respond to a performance-promoting supplement when they are housed under clean, disinfected conditions and moderate stocking density.

Deng et al [5] were investigating the effect of an aqueous alfalfa extract (AAE) on production, performance and egg quality of laying hens between 28 and 36 weeks of age. Dietary AAE had no effect on egg production parameters but shell strength was increased with gradient addition of alfalfa extract. Moreover, egg shape index, Haugh unit, egg albumen index and egg yolk index were unaffected by AAE supplemented diets.

Conclusions

Summarizing, the results show no effect of examined feed additives on performance and most of the egg traits, but it cannot be excluded that positive effects would have been observed in long-term experiment or under less hygienic environmental conditions.

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Growth Performance of Piglets Fed Pharmacological Dosage of the Regular Form and Low Dosage of a New Potentiated Form of Zinc Oxide

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Summary: One hundred and forty piglets (L × Y × D) were randomly allotted into 4 groups to determine the effects on piglets receiving either pharmacological dosage of ZnO or a low dosage of HiZox. Dietary treatments were: NC, negative control, basal diet containing Zn from the premix; PC, positive control, NC + 3,000 ppm ZnO; Hiz1, NC + 3,000 ppm ZnO (phase 1, 1 – 2 week)/200 ppm HiZox (phase 2, 3 – 6 week); Hiz2, NC + 300 ppm HiZox (phase 1)/200 ppm HiZox (phase 2). All supplemented groups showed better fecal scores than NC, without differences between PC and HiZox groups. At 2 weeks, average daily gains (ADG) were higher in the PC and HiZox groups than in NC. During phase 1, there were numerical improvements in favour of supplemented groups. For the whole period, NC gave the lowest ADG and Hiz1 the highest value, with PC and Hiz2 as intermediate results. The activity of alkaline phosphatase was improved ($P < 0.05$) in PC, Hiz1, and Hiz2 treatments compared with NC treatment at week 2. The plasma Zn concentration was higher ($P < 0.05$) in PC, Hiz1, and Hiz2 treatments than NC treatment at week 2. The fecal *Lactobacillus* concentration was lower ($P < 0.05$) in NC than other treatments at 2 week of this trial. Results from this study showed that HiZox was able to replace the regular zinc oxide at a dramatically reduced supplementation level.

Introduction

Zinc deficiency reduces serum zinc, alkaline phosphatase and albumin levels, and then depresses growth performance [1, 2]. The bioavailability of zinc depends on the source and dose of dietary zinc.

According to NRC recommendation [3], the recommended zinc level is 100 ppm in piglet feed. However, 2,000 to 4,000 ppm zinc are commonly supplemented in piglet diets. Pharmacological concentrations of ZnO have been proven to be effective in promoting post-weaning pig growth performance [4] and reducing the incidence of diarrhea [5], but would cause Zn pollution in the environment. Alternative and more sustainable practices are studied. A new form of potentiated zinc oxide (HiZox, Animine) showed higher ability to inhibit bacterial growth with *in vitro* and *ex vivo* conditions [6]. Therefore, this trial was conducted to investigate the different effects of ZnO and HiZox in weanling pigs.

Material and methods

Animals

A total of 140 weanling pigs [(Y × L) × D] with an average BW of 6.50 ± 1.11 kg were used in a 6-week experiment. Pigs were randomly allotted to 4 treatments with 7 replicate pens and 5 pigs per pen according to their initial BW.

Dietary treatments

NC: negative control, basal diet contains Zn from premix; PC: positive control, NC + 3,000 ppm ZnO; Hiz1: NC + 3,000 ppm ZnO (phase 1)/200 ppm HiZox (phase 2); Hiz2: NC + 300 ppm HiZox (phase 1)/200 ppm HiZox (phase 2).

Table 1 Zinc concentration in experimental diets

Zinc, ppm	NC	PC	Hiz1	Hiz2
Phase 1	148	2,483	2,498	351
Phase 2	131	2,429	237	225

Sampling and measurements

The ATTD of DM, N and E was measured following the procedures outlined by the AOAC [7]. The WBC, RBC and lymphocyte levels were determined using an automatic blood analyzer (ADVIA 120, Bayer, NY), and the immunoglobulin G (IgG) concentration using an automatic biochemistry blood analyzer (HITACHI 747, Hitachi, Tokyo, Japan). The alkaline phosphatase (ALP) activity and the plasma zinc was separately measured by the method previously described [8].

Procedures of microbial shedding and fecal score

In vitro survival of *Lactobacillus* and *E. coli* was determined according to the methods of Guo [9] and Mikkelsen [10] with certain modifications. The fecal score was determined by the average value of five pigs of each pen by using a 5 grade score system.

Statistical analysis

ANOVA was performed using the GLM procedure of

SAS (SAS Inst. Inc., Cary, NC).

Results and discussion

Growth performance and apparent total tract digestibility of nutrient

The ADG was lower ($P < 0.05$) in NC treatment than other treatments in the first 2 weeks, and increased ($P < 0.05$) in Hiz1 treatment compared with NC treatment all over the periods. The ADFI and G/F were not affected by dietary treatments, which was in agreement with the unimproved nutrient digestibility. The reason for the differing time responses to supplemental zinc is unclear. Carlson et al. [11] reported that both early-and later-weaned pigs also responded in a beneficial manner to supplemental ZnO from weaning to 14 d post-weaning with minimal differences in responses thereafter.

Table 2 Effect of HiZox supplementation on growth performance in weanling pigs

Items	NC	PC	Hiz1	Hiz2	SE
0 ~ 2 week					
ADG, g	283[b]	310[a]	316[a]	318[a]	6
ADFI, g	371	373	377	380	22
G/F	0.763	0.828	0.838	0.837	0.046
2 ~ 6 week					
ADG, g	474	504	513	506	15
ADFI, g	676	681	682	696	25
G/F	0.701	0.74	0.752	0.727	0.041
Overall					
ADG, g	411[b]	437[ab]	447[a]	443[ab]	9
ADFI, g	495	500	499	503	20
G/F	0.83	0.874	0.896	0.881	0.045

[a,b] Means in the same row with different superscripts differ ($P < 0.05$).

Blood profiles

The ALP activity and plasma Zn concentration were lower ($P < 0.05$) in NC treatment than other treatments at 2 weeks. Carlson et al. [11] demonstrated that plasma Zn concentration increased as the dietary concentration of Zn increased. The form of Zn in the diet can affect its bioavailability and subsequent plasma Zn concentration. The PC and Hiz1 treatments have no significant differences with treatment Hiz2, however biomarkers have probably plateaued.

Table 3 Effect of HiZox supplementation on blood profiles in weanling pigs

Items	NC	PC	Hiz1	Hiz2	SE
2 week					
IgG, mg/dL	440	390	408	402	74
ALP, U/L	357[b]	441[a]	459[a]	426[a]	18
RBC, $10^6/\mu l$	6.49	6.24	6.36	6.37	0.22
WBC, $10^3/\mu l$	19.06	19	18.01	18.7	1.84
Lymphocyte, %	49.7	47.5	51.7	55.2	4.2
Plasma Zn, μg/dL	140[b]	166[a]	170[a]	176[a]	8

[a,b] Means in the same row with different superscripts differ ($P < 0.05$).

Fecal microbial shedding and fecal score

In our study, the fecal *E. coli* count was not affected by the supplementation ofZn. After 2 weeks, the *lactobacillus* count was increased ($P < 0.05$) in all supplemented groups, and HiZox supplied at low dosage showed the same effect than pharmacological dosage of regular ZnO. At 6 weeks, the duration is long enough to neutralize the effects of dietary manipulation on gut

Table 4 Effect of HiZox® supplementation on fecal microflora in weanling pigs

Items, $\log_{10}$cfu/g	NC	PC	Hiz1	Hiz2	SE
2 week					
Lactobacillus	6.37[b]	7.41[a]	7.46[a]	7.66[a]	0.19
E. coli	5.49	5.31	5.3	5.24	0.18
6 week					
Lactobacillus	5.61	6.53	6.46	6.78	0.52
E. coli	4.92	5	4.7	4.96	0.27

[a,b] Means in the same row with different superscripts differ ($P < 0.05$).

microbiota. Previous research proved that high dietary concentrations of ZnO reduced post weaning pig mortality and diarrhea [12]. Subsequent research with weanling pigs demonstrated increased pig growth responses when high concentrations of ZnO were fed to weanling pigs without conditions of diarrhea [11].

At the end of weeks 3 and 4, the fecal score of treatment NC was higher ($P < 0.05$) than the other treatments, which could be explained by the higher plasma Zn at the end of 2 weeks because of the report of Huang et al. [13] that supplementing the starter diet with 3000 ppm zinc oxide for weaned pigs during the first week after weaning may reduce the numbers of bacteria reaching the ileal mesenteric lymph nodes. At the end of week 1, only treatment Hiz2 exhibited less soft feces ($P < 0.05$) compared with NC treatment.

Our results therefore suggest that HiZox, supplemented at low dosage in substitution of the pharmacological dosage of the regular zinc oxide, can improve growth performance and prevent scours in weaned piglets.

Table 5 Effect of HiZox supplementation on fecal score in weanling pigs

Items[1]	NC	PC	Hiz1	Hiz2	SE
Initial	3.7	4	4	3.7	0.2
1 week	4.0[a]	3.3[ab]	3.3[ab]	3.0[b]	0.3
2 week	3.7	3.3	3	3	0.2
3 week	3.7[a]	3.0[b]	3.0[b]	3.0[b]	0.2
4 week	3.7[a]	3.0[b]	3.0[b]	3.0[b]	0.2
5 week	3	3	2.7	2.3	0.2
Final	2.7	2.3	2	2	0.2

[1] Fecal score: 1 = hard, dry pellets in a small, hard mass; 2 = hard, formed stool that remains firm and soft; 3 = soft, formed, and moist stool that retains its shape; 4 = soft, unformed stool that assumes the shape of the container; 5 = watery, liquid stool that can be poured.
[a,b] Means in the same row with different superscripts differ ($P < 0.05$).

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Prevention of Swine Intestinal Disorders with Fitobiotics

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Summary: At the Vienna Congress of our Society we gave account on the development of an effective method for extracting the active substances from medicinal plants against enteric disorders of growing-finishing pigs. The most effective concentrations of the herb extracts were used for pilot studies. On basis of the very promising preliminary studies a feed additive (Bio-Santrix) was formulated and used in further trials to check its efficiency against enteric disorders of pigs. These novel data unanimously prove that the additive in adequate feed concentration abolishes or considerably alleviates the clinical manifestation of enteric disorders including swine dysentery, intestinal spirochetosis and porcine proliferative enteritis. If in spite of proper use of the additive, any form of the above disorders appear a single traditional treatment with antibiotics will yield dramatic improvement. On this basis medical expenses are reduced. Due to decreased rate of individual treatments labour intensity is eased, the feed-to-gain ratio improved and short return of medication costs is guaranteed. Further to this the additive is not liable for veterinary control, no medical-resistance forms against the active substances and the residues (if any) in the carcase might improve the culinary value of the lean and fat tissue.

Introduction

Medicinal plant extracts known as essential oils are fragrant and volatile ingredients of spices and herbs and have long history of use in traditional (folk) medicine. Anti-inflammatory, antioxidant, antimicrobial, antifungal, flavouring, (positive/negative) allelopathic and pesticidal properties of essential oils, herb and spice mixtures have been well documented. Related agricultural research gathered momentum by banning the use of antibiotics as production promoters and by the concern about the ever increasing resistance against antimicrobial drug preparations.

Most important enteric diseases of pigs involve intestinal spirochetal infections and the porcine proliferative enteritis. Of the former group swine dysentery caused by Brachyspira hyodisenteriae prevails in 45% of the Hungarian large-scale farms (Biksi et al., 2007). The causative agent proliferates in the large intestine of pigs degrading the surface and provoking excessive mucus and blood secretion. Diarrhoea is the prominent clinical symptom with large amount of mucus and occasionally flecks of blood in the faeces with consequent dramatic reduction of body weight, especially if the condition is left untreated. The aetiological agent of intestinal sprichetosis is the B. pilosicoli which apart from pigs may occur in wide range of hosts. Affected pigs discharge mucus containing non bloody diarrhoeal faeces with consequent depressed growth rate and poor feed conversion. Porcine proliferative enteropaties (PE) involve acute and chronic enteric disorders with variable clinical symptoms and with a common pathological picture: thickened mucosa of the small intestine and colon. The cause of PE is the Lawsonia intracellularis, an obligate intracellular bacterium which might cause (mostly moderate) diarrhoea. When present the faeces is loose, sloppy to watery with greyish-greenish colour. In chronic cases the stool does not contain mucus or blood.

The foregoing enteropaties have been demonstrated to have housing and management implications. Increased infective pressure as consequence of negligence of using the all in all out principle or routine disinfection, inefficient cleaning and disinfection methods, or use of housing systems (e. g. slatted floor) that presents difficulties in efficient cleaning and disinfection contribute to the clinical manifestation and recurrence. Excessive prevalence of environmental stressors or presence of immunsuppressive materials (e. g. T-2 toxin) in the feed has also been associated with the appearance enteric disease of swine. It follows; any medication programme should be used in conjunction with management practices that reduce the microbial load and eliminate stressors and immune suppressive materials. Immune enhancement might be supportive to antimicrobial treatments and prevention of the enteric diseases.

This paper discloses the main results of field trials

conducted with the feed additive (Bio-Santrix®).

Material and methods

Five trials were conducted in pig farms notorious for high prevalence of swine dysentery. In these farms parallel groups of pigs were formed at the beginning of the fattening period. The groups were housed within the same fattening house with identical conditions. Feeds of the control and experimental pigs were identically formulated but the diets of the experimental pigs were supplemented with Bio-Santrix® at 2 kg/ton concentration.

The effect of the feed additive on swine dysentery was measured by the cost of medication. Other additional effects were analysed by data of daily weight gain and feed conversion efficiency.

Results and discussion

Table 1 shows the number of pigs, feed to gain ratio (FCR) and rate of culling and mortality according to farms. Culling and mortality was low in all farms and had no effect on the value of the feed supplement. Data of feed conversion efficiency were within the range characteristic for the Hungarian pig farms. It is noteworthy; pigs of the treatment groups produced from less feed 1 kg weight gain. The difference varied in the range of 0.14 – 0.26 kg/kg which might be considered considerable. In a small farm (Farm No. 1) the difference was as big as 0.72 kg/kg.

Table 1

Farm	Item	Control	Experimental	Difference
No. 1	Number of pigs	20	19	
	FCR, kg/kg	3.89	3.17	0.72
	Culling and mortality, %	0	0	0
No. 2	Number of pigs	90	82	
	FCR, kg/kg	3.36	3.22	0.14
	Culling and mortality, %	0	0	0
No. 3	Number of pigs	331	326	
	FCR, kg/kg	3.31	3.1	0.21
	Culling and mortality, %	0.024	0.018	0.006
No. 4	Number of pigs	423	421	
	FCR, kg/kg	3.37	3.2	0.17
	Culling and mortality, %	0	0	0
No. 5	Number of pigs	440	441	
	FCR, kg/kg	3.22	2.96	0.26
	Culling and mortality, %	0.045	0.034	0.011

The weight increment of the pigs are characterised by the daily weight gain and illustrated in Fig. 1. It is seen that experimental pigs had somewhat faster growth rate in comparison with the controls. The difference ranged from 4 (Farm No. 5) to 39 (Farm No. 3) g/day·pig. These small differences are on the one hand negligible from practical point of view and are statistically not significant. However, figures indicate tendentiously and consistently better weight increment of the treatment groups.

The efficiency of feed additives known to have capacity against enteral disorders of pigs might be best illustrated by the combined cost of medication and prevention. In the present experiment the cost of treatments with traditional antimicrobial agents and cost of Bio-Santrix incorporated into feeds was recorded and calculated for 1 kg weight gain. Relevant data are shown in Fig. 2.

In the first two farms (Farm No. 1 and No. 2) there was no difference between the control and experimental pigs. This might be explained by the sporadic clinical manifestation of swine dysentery and ileitis and by the individual treatment of the pigs. In the other three farms beside individual treatment of the sick animals the feeds of the control pigs were occasionally medicated with antibiotics. In these farms the difference between cost of medication and prevention of experimental and control pigs amounted to 0.79, 1.3 and 0.77 cent/kg weight gain in Farms 1, 3, 4 and 5, respectively.

The findings of the present investigation have shown good agreement with other experiments reported in the relevant literature. Herbal extract (based on thyme, clove and oregano at levels of 0, 700, 1400 and 2100 ppm) decreased the diarrhoea incidence and improved the live weight gain of pigs (Pedroso et al., 2005). Piglets fed rations containing sage, coriander, yarrow and thyme were reported to have significantly higher daily gain (by 7%) and feed conversion efficiency (by 3%) than controls (Wagner, 2003). The inclusion of essential oils (at 25 mg/kg) of oregano, or thyme or garlic in the pigs' diet significantly improved the average daily weight gain and feed conversion ratio as compared to control pigs and

also had positive effects on the carcase composition (Onibala et al., 2001). Plant extracts composed of pepper, cinnamon, oregano, thyme and aniseed can increase the digestibility of pig rations from 81 to 85%, with a concomitant 5% increase in piglet live weight gain (Mavromichalis, 2003).

Fig. 1 Daily weight gain of contorl and experimental pigs

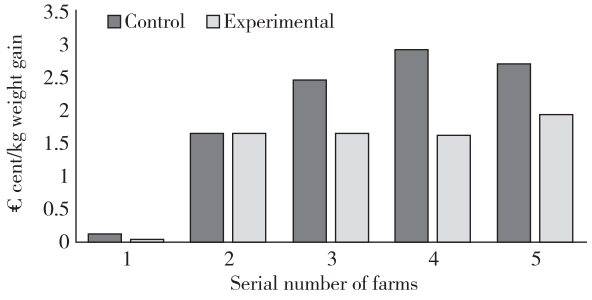

Fig. 2 Cost of medication and prevention

Conclusions

Experiments conducted with Bio-Santrix® supplemented feeds prove that its herbal extract ingredients are efficient and the active substances are present in appropriate combination. The feed additive when applied at 2 kg/ton concentration as suggested by the manufacturer

· abolishes or considerably alleviates the clinical symptoms of swine dysentery and iletis;

· reduces the cost of individual and farm level medical costs and expenses of prevention;

· and improves FCR, reduces the rate of culling and mortality and decrease the labour requirement.

On this basis, the feed supplement offers alternative method for substantial decrease of use of antibiotics in pig production. Further to this the use of the additive is not liable for veterinary control, no medical-resistance forms against the active substances and the residues (if any) in the carcase might improve the culinary value of the lean and fat tissue.

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The Effect of an Herbaceous Feed Additive and Zn-Bacitracin on Performance and Gut Morphology in Broilers Experimentally Infected with *Clostridium perfringens* Type A cpe-cpb2.

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Summary: Type A strains of *C. perfringens* are commonly found as part of the normal intestinal microflora of broilers and produce lethal and necrotising α toxins that are incriminated in necrotic enteritis in poultry. These strains carry a chromosomal enterotoxin gene (*cpe*) and in gastro intestinal diseases a gene associated with production of beta2 toxin (*cpb*2).

Laboratory trials were conducted with Cobb broilers kept on standard corn-soy diets. Four hundred-and-sixty, day old broilers, were assorted randomly into 4 groups of 115 with 5 replicates of 23 birds per group. The birds were kept in optimal environment in pens of 1.5 × 1.5 m embedded with wood shavings. Feed and water was available ad lib. from dispensers. Of the 4 groups' one negative and one positive control group was formed. No feed additive was added either of these groups. The feed of the 1st and 2nd experimental group was supplemented at 1 kg/ton concentration with an herbal preparation (Herbanoplex) and Zn-bacitracin, respectively. On day 18, 21 and 24 the birds of the positive control group and of the two experimental groups were inoculated orally with 2 ml of 10^8 CUF/ml inoculum containing Clostridium spp. Clinical signs were observed and data of production was collected and processed. Gross pathological findings of the small intestines were scored. Morphopathological measurements on villi and crypt of the small intestine were also included.

Mild to severe signs of necrotic enteritis developed in each treatment groups following the experimental infection. Of the bacteriologically challenged group, the one that received Herbanoplex additive in the daily ration proved in every respect superior to the other challenged groups.

Introduction

Necrotic enteritis (NE) is the most common and financially devastating bacterial disease in modern broiler flocks. It is an infectious disease caused by *Clostridium perfringens* (CP), which is a nearly ubiquitous bacteria readily found in soil, dust, faeces, feed, and used poultry litter. Of the five types of CP (A, B, C, D and E), which produce number of toxins (alpha, beta, epsilon, iota and CPE), Type A strains are commonly found as part of the normal intestinal microflora of broilers and may produce lethal and necrotising α toxins that are incriminated in necrotic enteritis in poultry. These strains carry a chromosomal enterotoxin gene (*cpe*) and in gastro intestinal diseases a gene associated with production of beta2 toxin (*cpb*2). CP can cause NE when it transforms from non-toxin producing type to toxin producing type.

NE is a multifactorial disease. The enterotoxemia that results in clinical disease most often occurs either following an alteration in the intestinal microflora (dysbiosis) or from a condition that results in damage to the intestinal mucosa (e. g., coccidiosis, mycotoxicosis, salmonellosis, ascarid larvae). High dietary levels of animal by-products (e. g., fishmeal), highly viscous cereal grains (wheat, barley, oats, or rye) predispose birds to the disease. Anything that promotes excessive bacterial growth and toxin production or slows feed passage rate in the small intestine could promote the occurrence of necrotic enteritis.

Maintenance of intestinal integrity is a critical component of prevention and control of NE. Integrity is not only the prevention of intestinal lesions. Rather, it is optimization of the process of digestion and absorption of nutrients and protein turnover, the physical and physiologic integrity of the epithelial barrier and the microbial ecology of the intestinal content (Wilson et al., 2005). Control measures, therefore, should be based on managing known risk factors for NE and the use of approved antimicrobial agents with proven efficacy against CP (Wilson et al., 2005).

Incidence of CP-associated necrotic enteritis in poultry has increased in countries that stopped using antibiotic growth promoters (Van Immerseel et al., 2004). Therefore use of feed additives that prevent dysbacteriosis and aid maintaining intestinal integrity has gained importance recently.

The goal of the present investigation was to check the efficiency of an herbaceous feed additive (Herbanoplex) in prevention of clinical manifestation of NE following artificial infection of broilers with Clostridium perfringens

Type A cpe-cpb2-.

Material and methods

Four hundred and sixty day old Cobb broilers were randomly assembled into 4 groups of 115 birds per group. Chicken groups with 5 replicates of 23 birds per group were kept in pens of a climatically controlled chamber with wood shaving beddings. Drinking water was provided from Plasson drinkers, feed was offered ad lib. first from plastic poultry feeders later from galvanised troughs. Known amount of feeds were weighed in the feeders at 9.00 a.m. daily and the quantity of leftover was collected and taken with an electronic balance (± 1 gramm). Climatic requirement of the birds (incl. ambient temperature, rel. humidity and air velocity) was fully met throughout the 42 days of the experiment.

All four groups received the same standard corn-soy diet (12 700 M.E./kg; 20.35% crude protein; 1.2% total lysine; 0.88 total meth. + cyst.; 1.02% calcium; 0.45% available phosphorus and 0.19% sodium) that contained anticoccidial (Diclaruzil; 0.2 kg/tonne) till 21 day of age of the birds. Of the 4 groups Group 1. served negative control, viz. the birds consumed the basal diet throughout the experiment. Group 2. (positive control) had the same feeding regime, however, the birds were orally challenged on day 18, 21 and 24 with 2 ml of 10^8 CFU/ml inoculum containing Clostridium spp. Pullets in Group 3 and 4 were challenged identically. The basal diet of these groups was supplemented with Herbanoplex CP (1 kg/tonne) and Zinc bacitracin (1 kg/tonne), respectively.

The birds were observed daily by visual inspection and by video camera. Special care was taken to identify prostrate, depressed birds with diarrhoea and appearance of blood-stained or orange-coloured faeces; lameness; ruffled feathers and discoloration of the skin on legs of broilers.

At 42 days of age 9 birds per treatment were randomly selected, electrically stunned and exsanguinated with an electric knife for evaluation of intestinal lesions. Macroscopic lesions in the small intestines from the duodenum till the terminal part of the ileum were scored according to Shojadoost et al (2012). In this score system 0 stands for integrity without gross lesions. Other scores indicate: thin or friable wall of the small intestine, or diffuse superficial but removable fibrin on the wall (Score 1); one to five focal necrosis or ulceration, or non-removable fibrin deposit (Score 2); less than 15 focal necrosis, or ulceration, or non-removable fibrin deposit (Score 3); 16 or more focal necrosis or ulceration, or non-removable fibrin deposit (Score 4); patches of necrosis 2 to 3 cm long (Score 5) and diffuse necrosis typical of field cases (Score 6). Post mortem examination also included recording the dead weight of the birds and weights of thymus, Bursa Fabricii and spleen. Two cm long samples were taken from the middle part of the duodenum, jejunum and ileum for histopathological examination.

Of the production parameters live weight of birds were taken weekly; on basis of the daily feed intake the weekly FCR (feed conversion rate, kg/kg) was calculated; mortality was recorded daily and the European Efficiency Index (EEI) was evaluated at the end of the experiment.

The results were analysed using the Statistical Analysis System (SAS, 1999) per experimental unit (replicate) using variance analysis (ANOVA).

Results and discussion

Challenging the birds with *C. perfringens* inoculate developed strong clinical signs of NE in Group 2 (positive control) by 28 – 30 days of age. The faeces became watery with undigested feed particles and the colour turned light brown. Five-seven days later the birds of this group became more and more aggressive; toe and feather pecking appeared and the rate of mortality increased to 6.09% by the end of the experiment. Birds in Group 4 produced also watery faeces; however no aggressive behaviour was seen and only one bird was lost (0.87%). Neither negative control birds, nor birds treated with Herbanoplex (Group 3) showed the above clinical signs and no birds were lost in these two groups.

No macroscopic lesions of the small intestine were found in birds of Group 1. However, the unprotected birds of Group 2 had variety of lesions from thin flaccid intestinal wall, through signs of local necrosis, to extensive multifocal areas of mild and severe necrosis. The overall score of the 9 birds from Group 2 was 3.8. Treatment with Herbanoplex substantially reduced the lesions characteristic for NE. The average score of the 9 birds of this Group (1.2) indicated presence of friable walls and presence of removable fibrins with sporadic appearance of focal necrosis. Zn-bacitracin in the feed of the Group 4 broilers alleviated also the gross pathological signs with an average score of 1.8.

Results of morphometric evaluation of the small intestine are disclosed in Table 1. Length and width of the villi is a good indication of the absorptive capacity. The height and width of the villi and depth of the crypts

Table 1 Morphometric evaluation

Group	Villus length(μm)	Villus width(μm)	Depth of crypts(μm)	Villus/crypt ratio
Group 1	2420	238	343	7.0
Group 2	1850	156	210	8.8
Group 3	2350	241	351	6.7
Group 4	2240	217	336	6.7

in the small intestine of Group 2 birds showed remarkable decrease in comparison with the negative control birds. Incorporation of Herbanoplex or Zinc bacitracin into the diet prevented this dramatic decrease indicating beneficial effects of the treatments on the regenerative capacity of enterocytes.

Weight gain of the broilers was identical among the four groups till the time of the bacterial challenge. After inoculation the weight gain of birds in the positive control group gradually lagged behind the other three groups (Fig. 1). At conclusion of the experiment the live weight of broilers in Group 2 (2225 ± 21.3 g) was significantly ($P \leq 0.05$) inferior to groups 1, 3 and 4 (2610 ± 15.2; 2643 ± 18.3 and 2595 ± 16.3, respectively). It is noteworthy that the Herbanoplex group produced the best final live weight. The total feed consumption per bird was 4477, 4520, 4450 and 4480 g/bird in Group 1, 2, 3 and 4, respectively. The overall feed conversion efficiency of the four groups was 1.71 kg/kg (Group 1) and 2.03, 1.68, 1.73 in Groups 2, 3 and 4, respectively.

The overall efficiency of broiler production is best characterised by the *European Production Index* (*EPI*), which is calculated as quotient of net product of rate of survivor birds multiplied with the average live weight at conclusion of the production period and net product of days of production multiplied with the feed conversion rate. In this experiment this index was 363.45, 254.44, 374.58 and 357.17 in Groups 1, 2, 3 and 4, respectively. The best index was found with the Herbanoplex group and the figure (374.58) was statistically different ($P \leq 0.05$) from all groups investigated in the present experiment. Statistical analysis showed that *EPI* of the positive control group was inferior to all of other groups.

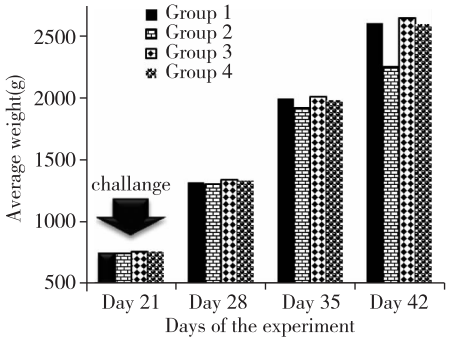

Fig. 1 Average weight of birds

Conclusion

Management of disease associated with CP should focus on prevention and, in cases where this fails, early detection and treatment. Prevention strategies should include minimizing exposure to known risk factors. Prudent use of feed additives with demonstrated efficacy against CP is also recommended and, in some cases, essential for the prevention of NE or treatment of outbreaks when they occur (Wilson et al., 2006). Data of the present experiment are convincing for the efficiency of Herbanoplex in its preventive capacity of NE of broilers.

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Preventive Application of Propolis and Herbs Extract in Rabbits Experimentally Infected with Enteropathogenic *E. coli* Strains

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Summary: The aim of the study was to determine an influence of ethanolic extract of propolis (EEP) and herbal extract in rabbits experimentally infected with *E. coli* on antioxidative status, biochemical blood parameters and health status.

An application of ethanolic extract of propolis (EEP) in rabbits infected with *E. coli* strains limited number of collapses and positively affected body weight gains. No *E. coli* presence in small intestine was noted in case of experimentally infected rabbits supplemented with EEP. Long-term administration of an extract of examined herbs may be related to an unprofitable influence on liver secretory functions. Positive influence of EEP on blood antioxidative status was noted.

Introduction

Diarrhea and collapses caused by enteropathogenic *E. coli* strains (EPEC) are the main reason of economic losses in rabbit broilers production. Losses caused by these strains ranges from 20% to 40%. Currently, main emphasize is put on treatment of disease cases with antibiotics application. Such action leads however to many negative consequences. Taking into account specificity of rabbits alimentary tract activity, antibiotic application gives very differentiated effects, causing in some cases even deterioration of clinical state and increase in collapses. Concurrently, such an activity contributes to formation and selection of bacterial strains resistant on antibiotics.

In a view of an increasing drug-resistance of microorganisms, decrease in an amount of antibacterial chemotherapeutics used would be undoubtedly a large benefit. The decision of European parliament establishing the second community program of activities in the field of health for the years 2008 – 2013, where microorganisms resistance on antibiotics and therapeutic limitations of infections are listed among priority threats of public health, proves the significance of this problem. Probiotics, prebiotics, herbs as well as propolis are an alternative for antibiotics [1 – 3].

The aim of the study was to determine an influence of ethanolic extract of propolis (EEP) and herbal extract in rabbits experimentally infected with *E. coli* on antioxidative status, biochemical blood parameters and health status.

Material and methods

The study was performed on rabbits (Hyplus line) starting from weaning. Clinically healthy rabbits were attributed to particular groups (n = 12): group Ⅰ – control; group Ⅱ – experimental (10% ethanolic extract of propolis was added to water in amount of 2 ml/l water); group Ⅲ – experimental (10% extract (20 ml/l water) from *Rumex cissus*, *Pontentilla anserina*, *Polygonum aviculare* herbs wt/wt 1∶1∶1). After 2 weeks of additives application the rabbits were infected *per os* with enteropathogenic *E. coli* strains isolated from farm rabbits with diarrhea (infectious dose 5×10^4). Blood analysis were performed at the day of beginning of the experiment and after 2 and 4 weeks. Hematological parameters, acid-base balance, biochemical parameters and antioxidative status (TAS and GPX) were examined in the blood. Body weight, fodder and water intake were registered during the experiment. Dissection and bacteriological examinations were conducted in collapsed rabbits, the samples were collected from small intestine and cecum.

The data was statistically processed with the computer program SAS, calculating mean values ($\bar{x}$) and standard deviations (SD). The significance of mean differences between the groups and within the groups between successive samplings was estimated with the Duncan test for each parameter studied.

Results and discussion

On commercial rabbit farms, mortality and culling are of great relevance from the production and financial

viewpoint. The highest risks of mortality and culling in does occurred during the first three kindlings, but remained stable thereafter. According to Rosell and de la Fuente [6] rabbits mortality resulting from an occurrence of alimentary tract diseases reaches 27%.

Rabbits from group Ⅱ receiving EEP obtained the highest body weight, while the lowest one was noted in the control group. Also the highest body weight gains were noted in group Ⅱ (Table 1). Higher body weight gains were noted in another nutritional study conducted on rabbits [5], however in our study feed intake decreased clearly after infection.

After 2 weeks of herbal extract application, an increase in total protein content, AST activity in blood was observed, while an increase in total antioxidative ability was noted in the EEP group. The highest activity of glutathione peroxidase was observed in the group receiving propolis at the day of the end of experiment (results not presented in the tables). After 4 weeks of herbal extract application, an activity of AST, GGT and total bilirubin concentration was the highest in group Ⅲ when compared to other groups. AST activity was also elevated in the group supplemented with propolis (Table 2). Oliveira et al. [4], reported that crude propolis extract did not cause significant alterations in the serum aspartate aminotransferase activities.

Table 1 Growth performance in rabbit

Groups	Initial live weight(g)	Final live weight(g)	Daily weight gain(g)
Control(Group Ⅰ)	1860.00 ± 203.2	2478.18 ± 245.0[a]	25.76 ± 2.8
Propolis(Group Ⅱ)	1808.90 ± 187.3	2624.02 ± 198.7[b]	33.96 ± 3.9
Herbs(Group Ⅲ)	1822.33 ± 167.9	2565.13 ± 134.58	30.98 ± 4.7

[a, b] – statistically significant differences ($P \leqslant 0.05$) between groups.

Very abundant growth of enteropathogenic *E. coli* strains was noted in bacteriological cultures from small intestine and cecum in collapsed rabbits. In microbiological examinations performed after the end of the experiment in rabbits subjected to euthanasia, an abundant increase in *E. coli* was noted in cecum in all the groups, however it was differentiated in small intestine. Sparse *E. coli* was noted in small intestine of 3 rabbits in group Ⅰ, no growth was observed in group Ⅱ, and sparse E. coli were detected in 2 rabbits in group Ⅲ. The ethanolic extract of Egyptian propolis, when administrated in combination with formalized inactivated *Pasteurella multocida* vaccine in rabbits' enhanced specific and nonspecific immune response [3].

Table 2 Selected biochemical blood indexes in serum blood of rabbits

Group	TP (g/l)	Albumin (g/l)	AST (U/l)	GGT (U/l)	Bilirubin (μmol/l)	Glucose (mmol/l)	LA (mmol/l)	Cholesterol (mmol/l)
				Start of experiment				
Control	49.68 ± 4.87	37.20 ± 3.56	25.81 ± 4.76	9.65 ± 1.65	2.69 ± 0.87	7.51 ± 1.67	16.86 ± 3.23	2.23 ± 0.78
Propolis	49.10 ± 3.89	36.8 ± 6.34	26.5 ± 4.89	8.3 ± 2.01	3.42 ± 0.76	7.40 ± 1.59	18.67 ± 3.27	2.38 ± 0.69
Herbs	47.80 ± 6.45	36.8 ± 9.68	26.2 ± 7.11	4.1 ± 0.67	1.54 ± 0.59	7.21 ± 1.45	12.97 ± 2.67	2.09 ± 0.67
				After 2 weeks				
Control	43.44 ± 3.56	35.82 ± 3.45	28.09 ± 4.56[A]	9.47 ± 6.78	3.03 ± 0.78	7.92 ± 2.34	12.77 ± 2.56	1.44 ± 0.87
Propolis	49.28 ± 7.32	36.12 ± 8.34	43.64 ± 8.98[B]	10.96 ± 3.44	3.93 ± 0.49	5.32 ± 1.87	12.39 ± 2.43	1.73 ± 0.67
Herbs	50.02 ± 3.98	35.16 ± 4.76	36.88 ± 4.56[C]	7.88 ± 3.65	3.84 ± 0.76	6.29 ± 1.57	11.62 ± 3.23	1.52 ± 0.59
				After 4 weeks				
Control	50.90 ± 4.56	37.08 ± 7.43	29.60 ± 5.98[A]	6.75 ± 1.89	2.75 ± 0.21	6.89 ± 1.58	14.76 ± 3.67	2.49 ± 0.68[a]
Propolis	53.04 ± 6.76	35.45 ± 5.76	47.09 ± 7.01[B]	5.88 ± 2.56	2.91 ± 0.34	6.22 ± 1.76	14.14 ± 2.67	1.69 ± 0.41[b]
Herbs	56.59 ± 4.67	35.05 ± 4.67	54.64 ± 5.78[C]	7.75 ± 1.43	3.19 ± 0.34	6.00 ± 1.98	14.71 ± 2.78	2.84 ± 0.43[a]

[A, B] – differences statistically highly significant ($P \leqslant 0.01$) between groups in given sampling; [a, b] – statistically significant differences ($P \leqslant 0.05$) between groups in a given sampling.

Conclusions

An application of ethanolic extract of propolis (EEP) in rabbits infected with *E. coli* strains limited number of collapses and positively affected body weight gains. Long-term administration of an extract of examined herbs may be related to an unprofitable influence on liver secretory functions. Positive influence of EEP on blood antioxidative status was noted.

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Serum Immunoglobulins Concentrations and Diarrhoea Symptoms in Calves Fed Milk Replacer with Propolis

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Summary: The aim of this study was to estimate the possibility of health improvement and the reduction of diarrhoea incidence in the neonatal period of calves by preventive use of the ethanol extract of propolis (EEP) as a fodder supplement.

The supplemented EEP had a positive effect for the examined calves, as the clinical symptoms of diarrhoea subsided. Due to the analysis of the concentration of separate immunoglobulin classes in the first weeks of life of calves, EEP was found to have protective action against infections. In contrast, in the group of calves that feed was not supplemented with EEP, immunoglobulin G_2 was 100% higher due to a pathogenetic factor, and suffered from diarrhoea symptoms.

Introduction

The main reason for the alimentary tract diseases (with diarrhoea as one of their symptoms) in calves in the neonatal period is the concurrence of numerous pathogens [5, 8]. Bacterial diarrhoea is managed with antibiotics, however, there is a growing number of reports on microorganism resistance to them [4]. Therefore, natural fodder supplements with anti-diarrhoea action and stimulation of the immunological system in calves, have become popular over the past several years [6, 12]. The reports on propolis preventive nature against diarrhoea and its stimulation of the immunological system in calves are scarce. Propolis preparations have been found to be effective in the prophylaxis of the diseases of the upper airways and stomatopathies in human and also in *in vitro* tests examining their bacteriostatic and germicidal properties [1, 10].

The aim of the study was to estimate the level of serum immunoglobulin concentration in the blood of calves as well as the possibility to limit the diarrhoea symptoms in the neonatal period by preventive supplementation of propolis.

Material and methods

The examinations were made on the group of 40 calves of the Holstein-Fresian breed, from the 2nd till the 20th day after birth. The newborns were divided on their 2nd day of life into 2 main groups, according to the following criterion/standard-γ-globulin level below (1) and above (2) 10 g/l (calves with lower and higher immunological status). Each of the main groups was divided into control groups (C1 or C2) and experimental groups of calves fed by an ethanol extract of propolis in amount of 3 ml after 7th day of calves life (P1 or P2). The EEP was supplemented in the morning by addition to the milk replacing preparation. The ethanol extract of propolis that was supplemented during this investigation is a dietary supplement allowed in Poland (registration number MZIOS NR-R/0275). The estimation of diarrhoea symptoms was made on the basis of clinical observation and evaluation of their excrements, according to the 4-point-scale (0 to + + +).

Blood samples were taken from the *vena externa jugularis* of each calf, on days 2 – 4, 7, 14 and 21 after birth. Blood serum was tested for: the level of total protein by means of the biuretic method and equipment manufactured by Cormay; the protein fraction – γ-globulins by the electrophoresis method on agar-agar gel in Cormay diagnostics chamber, with fraction reading in Cormay densitometer DS. -2; the levels of immunoglobulins of subclasses G_1, G_2 and M class, marked by the method of radial immunodiffusion after Mancini et al [9], using antibodies by Serotec and standards by Binding Site.

The data was statistically processed with the computer program SAS, calculating mean values ($\bar{x}$) and standard deviations (SD). The significance of mean differences between the groups and within the groups between successive samplings was estimated with the Duncan test for each parameter studied.

Results and discussion

The calves were examined for clinical symptoms of diarrhoea and its intensity during each blood sampling. In control groups diarrhoea symptoms were observed throughout the duration of the experiment, irrespective of a sufficient or insufficient supply of colostrum. In the experimental groups clinical symptoms of diarrhoea (weak or mild) occurred until the 7th day of life, and after that time the EEP cured these symptoms (Table 1). Once the EEP was supplemented to the animals, the symptoms

subsided. A positive influence of EEP against diarrhoea in calves at this age was also confirmed by Chudoba-Drozdowska et al. [3].

Table 1 Clinical diarrhoea symptoms

Groups	Day of life			
	2 – 4	7	14	21
	Calves with γ-globulins level below 10 g/l, on days 2 – 4 of life			
C1	0	+ +	+	+ +
P1	0	+	0	0
	Calves with γ-globulins level over 10 g/l, on days 2 – 4 of life			
C2	0/0	+	+ +	+
P2	+	+ +	0	0

The level of TP increased by day 7 of life in those calves, in which it was below 60 g/l on days 2 – 4 of life. The highest increase throughout the study period was observed in controls with low immunological status ($P \leqslant 0.05$) (Table 2). The increase of the TP level has been observed by Constable et al. [2] in animals with clinical symptoms of diarrhoea, which was confirmed in control groups. The level of TP and its decreasing tendencies during the first 3 weeks of life were the same as given by Knowles et al. [7], and only in groups C2 and P2 a satisfactory supply of lactoimmunoglobulins was achieved, reaching the standards defined for adult cattle [2].

Table 2 Selected biochemical blood indexes of the examined calves

Groups	Parameters				
	TP (g/l)	γ-globulins (g/l)	IgG1 (g/l)	IgG2 (g/l)	IgM (g/l)
	Sampling on days 2 – 4 of life				
C1	$^{*}47.32^{A} \pm 6.02$	$5.13^{A} \pm 3.09$	$5.08^{A} \pm 5.07$	$0.40^{A} \pm 0.3$	$0.70^{A} \pm 0.27$
P1	$47.36^{A} \pm 4.98$	$6.23^{A} \pm 2.96$	$6.21^{A} \pm 3.22$	$0.44^{A} \pm 0.14$	$0.67^{A} \pm 0.26$
C2	$62.39^{B} \pm 10.89$	$18.32^{B} \pm 9.45$	$^{*}14.98^{B} \pm 6.25$	$^{*}1.52^{B} \pm 1.03$	$0.97^{B} \pm 0.22$
P2	$60.38^{B} \pm 6.81$	$18.64^{B} \pm 5.87$	$^{*}18.38^{B} \pm 6.02$	$1.35^{B} \pm 0.33$	$1.33^{B} \pm 0.31$
	Sampling on days 7 of life				
C1	$48.85^{A} \pm 4.18$	$4.83^{A} \pm 2.6$	$4.66^{A} \pm 3.27$	$0.42^{A} \pm 0.37$	0.70 ± 0.31
P1	$47.8^{A} \pm 4.18$	$5.78^{A} \pm 1.99$	$6.31^{A} \pm 3.3$	$0.40^{A} \pm 0.23$	$0.61^{a} \pm 0.26$
C2	$60.8^{B} \pm 9.92$	$14.79^{B} \pm 7.97$	$^{*}14.03^{B} \pm 6.34$	$1.40^{B} \pm 0.59$	0.98 ± 0.49
P2	$60.4^{B} \pm 8.18$	$15.32^{B} \pm 4.9$	$16.60^{B} \pm 4.83$	$1.12^{B} \pm 0.19$	$1.24^{b} \pm 0.47$
	Sampling on days 14 of life				
C1	$^{*}53.17 \pm 6.39$	$5.11^{A} \pm 2.38$	$4.54^{A} \pm 3.09$	$0.39^{A} \pm 0.26$	0.65 ± 0.36
P1	$47.46^{A} \pm 5.81$	$5.54^{A} \pm 1.96$	$6.59^{A} \pm 3.62$	$0.40^{A} \pm 0.23$	0.63 ± 0.21
C2	$59.7^{B} \pm 10.3$	$12.82^{B} \pm 7.07$	$14.22^{B} \pm 6.15$	$1.22^{B} \pm 0.78$	0.81 ± 0.31
P2	$61.81^{B} \pm 5.85$	$13.5^{B} \pm 4.49$	$15.22^{B} \pm 6.37$	$1.14^{B} \pm 0.25$	0.86 ± 0.32
	Sampling on days 21 of life				
C1	$51.6^{A} \pm 5.78$	$5.30^{A} \pm 2.25$	$4.88^{A} \pm 3.35$	0.80 ± 0.64	0.46 ± 0.23
P1	$50.79^{A} \pm 4.91$	$5.44^{A} \pm 1.45$	$6.57^{A} \pm 3.35$	$0.53^{a} \pm 0.23$	0.72 ± 0.24
C2	$60.94^{B} \pm 8.66$	$11.36^{B} \pm 5.8$	$^{*}13.55^{B} \pm 3.88$	$^{*}0.95 \pm 0.55$	0.80 ± 0.36
P2	$59.64^{B} \pm 5.08$	$12.40^{B} \pm 3.72$	$^{*}13.42^{B} \pm 4.46$	$1.07^{b} \pm 0.32$	0.86 ± 0.12

* Significance of differences between samplings in the given group at $P \leqslant 0.05$.

$^{A, B}$ – differences statistically highly significant ($P \leqslant 0.01$) between groups in given sampling; $^{a, b}$ – statistically significant differences ($P \leqslant 0.05$) between groups in a given sampling.

In this study, the level of γ-globulins was decreasing during the investigation in groups C2 and P2 in contrast to the main group with a low level of γ-globulins. Moreover, the increase of the IgG2 concentration by 100% between 14th and 21st day of life, was caused by dysfunction of the alimentary tract, which stimulate calves to begin an earlier, non-physiological production of their own immunoglobulins. In the groups receiving EEP, individual immunoglobulin classes fluctuated within 20% when compared with their initial levels (days 2 – 4 of life). Chudoba-Drozdowska et al. [3] observed, applying propolis to calves with clinical symptoms of diarrhoea, a physiological decrease of γ-globulins level in individuals receiving the preparation, and it was considerably less intensive than in controls. In this research, the animals receiving the EEP, revealed changes in the γ-globulins level similar to those quoted above, which confirms a positive influence of the administered preparation. Takagi et al. [11] proved EEP to be influential on the activation of the immunological system of the mouse. EEP definitely suppressed the IgG production and stimulated IgM increase, activity of macrophages, secondary increase of cytoxical lymphocytes T number and interferon γ.

Conclusions

It was stated, that the supplemented ethanol extract of propolis had a positive effect for the examined calves, as the clinical symptoms of diarrhoea subsided. An increase of IgG2 concentration of about 100% by calves between 14th and 21st day of life was observed only in control groups. In groups of calves fed by propolis some fluctuations between particular immunoglobulin classes were noticed, though they were not exceeding 20% in relation to the initial level obtained on the 2nd day of animals life. The observed changes were a consequence of the protective effect of propolis on the alimentary tract of examined calves. Moreover, the frequency of clinical diarrhoea symptoms was reduced, on the contrary to calves of control groups.

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Antibacterial Evaluation of *Caesalpinia coriaria* Plant Extracts Against some Bovine Mastitis Pathogenic Bacteria

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Summary: The present work investigated the *in vitro* antimicrobial activities of methanolic and destilled water extracts corresponding to *Caesalpinia coriaria*, with common name Cascalote plant from Colima, México. 800 samples were taked at 200 cows, the California Mastitis Test was conducted. The samples were analysed *in vitro* for their biochemical isolation and identification in blood agar cultures. The antimicrobial activity was determinated by the method of Mueller Hinton agar diffusion. The used microorganisms were the bacteria *Staphylococcus aureus*, *Streptoccocus* spp. ,*Enterobactericea* group. Twelve investigated extracts both in methanol and water: Cascalote 100 and Cascalote Air both 100, 75, 50, 25 and 10 percent. Presented significant antimicrobial activity against *Staphylococcus aureus* 21 mm of inhibition zone, 23 mm for *Streptoccocus* and 21 mm for *Enterobactericeae* group. The extracts with the greatest antimicrobial activity were the extracts of Cascalote 100. The total soluble phenols test was analyzed by the Folin-Ciocalteu method, Tannins present in *C. coriaria* ranges of 40%. Phytochemical analysis of Cascalote revealed that the antibacterial activity is due to presence of phenolic fractions.
Key words: *Caesalpinia coriaria*, antimicrobial activity, plant extracts, Mastitis

Introduction

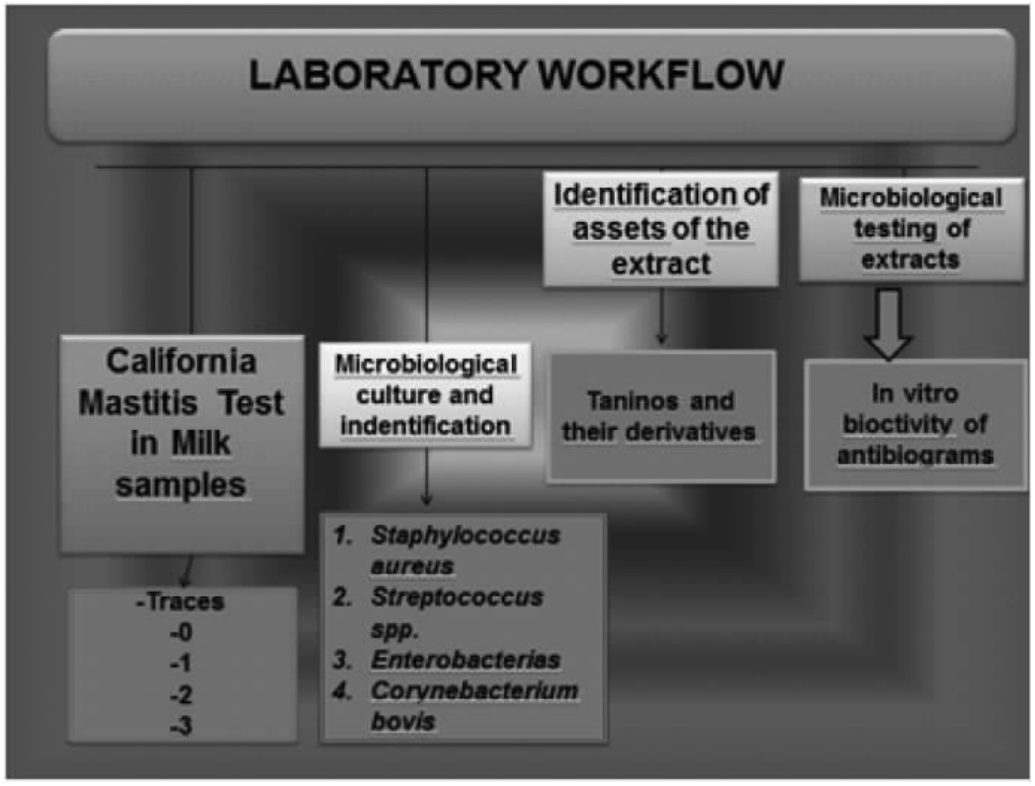
Bovine mastitis is the inflammation of the mammary gland, characterized by physical and chemical changes in milk and pathological changes in the glandular epithelium. Resulted of the interaction of several factors such as: management and hygiene animal during milking, cows susceptibility, environmental characteristics and the presence of microorganisms [1]. It may be clinical and subclinical o chronic. *Staphylococcus aureus* is the main agent of infectious mastitis [1]. Subclinical mastitis is difficult to detect affecting the physicochemical milk quality; is 10 – 40 times more common than clinical [2]. More than 400 years ago in México began milk production; currently has 31 million cattle, 9 million are used for milk production. The family production systems are 30 – 60 cows in Jalisco, is the most important; presents the dual purpose and large holdings from more than a thousand cows. The bacterial agents of bovine mastitis in México are: *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Corynebacterium bovis* and the *Enterobactericeae* group; which they are zoonotic to humans by the milk consumption [3]. *Caesalpinia coriaria* is a plant like a bush known as Cascalote, has been reported to be the fourth constituent in tannins from the kingdom *Plantae* [4], the pods have been used for the extraction of tannins and their concentration ranges between 40% – 42%; they are very resistant to microbial attack and inhibit growth of some microorganisms as the insects larval stage [5], as well as it has effect against the *S. aureus* methicillin resistant [6]. It is believed that their antimicrobial activity can be to their interaction on the adhesins and her ability to adhere to the polysaccharides [7]. In the tannins were found the inhibitory activity of the viral reverse transcriptase [8]. Them stimulates the phagocytic cells wrapping tumors cells [9]. They have shown the presence of triterpenoids that have been used for his strong antibacterial capacity, antineoplastic and other pharmaceuticals applications [9]; like a beutilinic acid inhibits the growth of HIV viruse. The terpenides or terpenes have activity against bacteria, viruses and protozoa.

No others investigations against bovine mastitis using Cascalote extracts, were reported. The Township Tlajomulco de Zúñiga, Jalisco has a prevalence of 31% of bovine mastitis and the main pathogen was found *Staphylococcus aureus* [10].

Material and methods

The work was developed in the mastitis and molecular diagnostic laboratory of CUCBA and the water quality and ground laboratory of ITTJ, Jalisco, México.

800 samples were obtained in 200 cows in differents breeds like Holsteins; in different dairy farm in localities/zones of Tlajomulco de Zúñiga, Jalisco. Respectively carrying out the California mastitis test like a selective analysis to discard the presence of bovine mastitis, subsequently the positive samples to the California mastitis test were seeded for 24 hours *in vitro* in blood agar-base for their isolation and biochemical identification and morphologically; after they were nourished at least 2 hours in brain-heart base-liquid. After they were inoculated and cultured for 24 and 48 hours in Mueller-Hilton solid culture agar; for the evaluation of the extracts bioactivity from *Caesalpinia coriaria*. The Watman filter paper was used from diameter 3 to 6 mm; we had to moisten in a filter paper by the different extracts concentrations.



The tannins isolations were for grinding and mesch filtration from the matures pods bush. We achieve to isolation of two differents substances according to consistence and particle size. They were named by ourselves: 1. Cascalote 100 and 2. Cascalote Air; they were extracted in distilled water and methanol. 20 extracts from Cascalote 100 and Air both in methanol, distilled water to: 100%, 75%, 50%, 25% – 10%; they were tested in different microbiological strains, the extracts bioactivity were measured in mm.

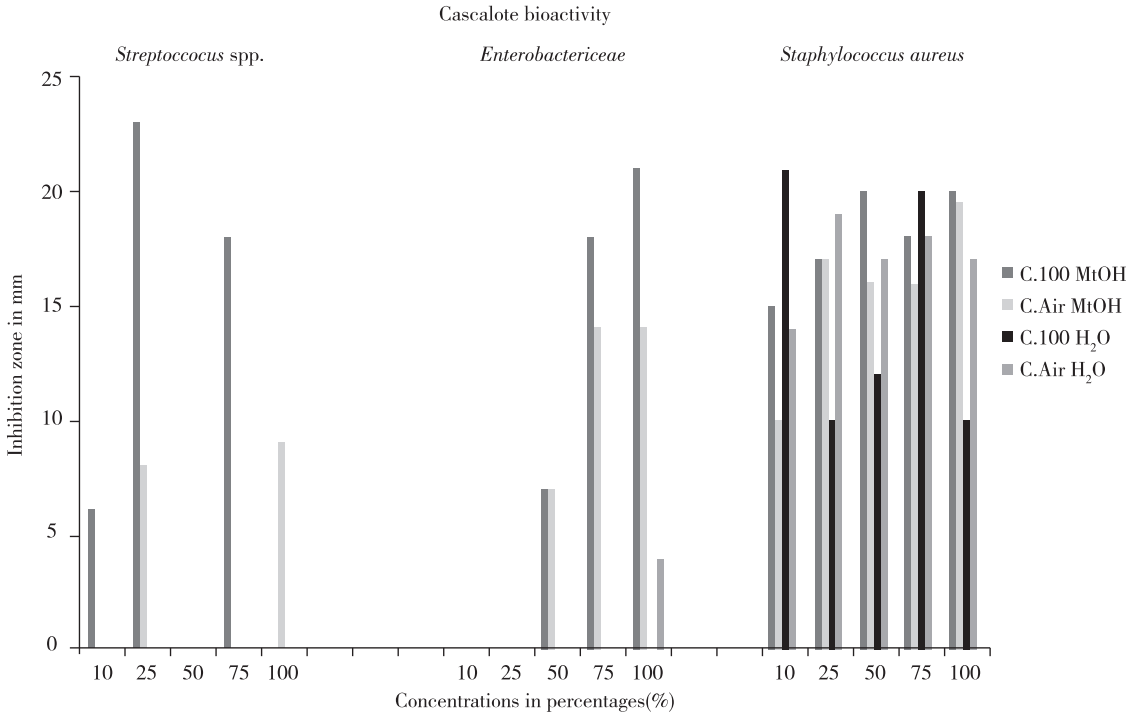
Results and discussion

The matures pods were recollected in the Colima City, they were milled and the fines powders were obtained with particles from ⩾ 100 μ. The crudes extracts tannins their total soluble phenols quantification by the method of Folin-Ciocalteu were 44.12%. The minimum inhibitory concentration (MIC) by the dilution method was determined. The inhibition zone was measured in mastitis bacterial cultures; the zones were obtained for: *Staphylococcus aureus* 21 mm, Enterobacterias 21 mm and 23 mm for *Streptococcus* spp..

The use of the extracts plants for treatments of the cows with mastitis, are limited; the phytodrugs diversity in the markets are scarces, alredy that phytodrugs are in experimental phase. By that reason we can to propose the Cascalote bioactivity against the mastitis bovine pathogens like a very important alternative in the treatments in these diseases. In the all experiments the results showed that all extracts from *Caesalpinia coriaria* have antibacterial activity against bacterial cows groups. The MIC was more effective from Cascalote 100 with methanol. We agree with the low effectiveness against *Streptoccocus* spp..

The extracts plants haven't presented any change with theirs respectives treatments; because the methanol and distilled water cannot inhibit the growing in the mastitis cows pathogens.

The disk diffusion test was used as an alternative



microbial susceptibility measure represented in millimeters (NCCLS, 1996). MIC (Minimum inhibitory concentration) was used for to determine the effective dosage against the bovine mastitis bacteria from Cascalote extracts in a series of dilutions in liquid culture tubes Brain-Heard. (NCCLS, 2000).

Conclusions

We suggest the biochemical identification of assets presents for discard a synergy maybe in all of them. We conclude that the Cascalote can be an effective alternative and cheap for control of the bovine mastitis and to avoid zoonotics diseases.

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The Effect of Sampling Methods on the Identification of *Staphylococcus aureus* in Cow Milk

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Summary: Mastitis is one of the most common diseases of the dairy cows, and it is responsible for huge economic losses in the dairy industry. From the many causing agents, *Staphylococcus aureus* is the most prevalent mastitis pathogen in Hungary. In case of a massive *Staphylococcus aureus* infection in a dairy herd, a preventive program is the only reliable solution which means that large number of milk samples must be examined to identify the infected animals. In the practice the current method is culturing these samples on agar plates. In our first experiment we examined how the long-term congelation can alter the microbiological results. We could culture the *Staphylococcus aureus* bacterium after one year, so the bacterium definitely can survive that maximum 1 – 1. 5 months what it spends in the farms deep freezer before the veterinarian sends it to the laboratory. On the other hand we also examined how the sampling procedure can affect the results. In two experiments, the effectiveness of pre-and post-milking composite samples, and individual quarter samples were compared. It is generally believed that post-milking samples are more reliable than the pre-milking ones, but it has turned out in both experiments, that the pre-milking composite milk samples are more effective to identify the infected animals than after-milking composite samples. We also found that culturing the same sample 3 times raises the accuracy while the examination of individual quarter samples cost four times more than composite samples and the results was only slightly better, so this method is unprofitable and not advised in practice.

Introduction

Staphylococcus aureus is one of the most prevalent mastitis pathogen bacterium, in our laboratory, 20% of the milk samples from dairy cows contains this bacterium. The occurring, mostly subclinical mastitis causes a huge economic loss just like the continuous culling of infected animals. The identification of these infected cows requires a constant monitoring of the herd. The most economic and practical way is the traditional microbiological examination of milk samples by culturing them on agar plates.

Material and methods

Our examinations had three parts. In the first one a *Staphylococcus aureus* infected udder quarter was sampled and the milk sample was divided into 13 identical parts. All of them were put in a deep freezer and were kept in –20℃. The next day the first one was removed from the freezer and after thawing on room temperature in was cultured on Columbia agar plate. The incubation was 48 h on 37℃. After this period the colonies were identified. Every month the same procedure was done with one of the frozen samples for one year. In the second part 13 infected cows were sampled. Composite milk samples were collected before the milking (but after the teat preparation and forestripping) and after it. Individual quarter samples were also collected after the milking. The samples were frozen for one night (–20℃) and were cultured on Columbia agar plates. The incubation time and temperature were like in the first experiment. The sampling was repeated on the same day in the next 5 weeks. In the third experiment the second one was reprised but with 25 animals for 5 weeks. And beside the previously mentioned samples a 1∶1 mixture was made from the two composite milk samples, and also from the quarter samples. The after milking composite sample was cultured three times.

Results and discussion

After one year in the deep freezer we still could culture the *Staphylococcus aureus* bacterium from the milk samples. It showed us, that in practice it is not a bad habit that farmers collect and freeze the milk samples continuously until they have enough to send it to a laboratory. The bacterium can survive in the farm's deep freezer until the sample is sent. In the second experiment from the 13 cows 2 were negative during the 6 weeks long sampling period and they were excluded from the analysis. The remaining 11 animals during the 6 week interval (66 examinations altogether) released the bacterium only in 42 cases (63. 63%), in these cases could we identify *S. aureus* from at least one milk sample. The pre milking composite samples produced 24 positive results which was only 36. 36%. It was a bit better if we look only those days when we could confirm the release of the bacterium (57. 14%). The post milking composite samples were the less effective in this

experiment with 22 positive results, which is only one third of all the cases (52.38% of the bacterium releasing days). Individual quarter samples were the most effective. With this method we could confirm the infection in 34 examinations, which is 51.52% (80.95% of the bacterium releasing days). From the third experiment we also excluded the constantly negative animals and those ones who produced only one positive sample. 18 animals remained for 5 weeks which is 90 examinations. The final number was 88 because two cows missed 1 – 1 sampling days. Altogether from the 88 examinations of infected animals, we could confirm the presence of the *S. aureus* bacterium in 68 cases (77.27%). On those days the cows were releasing the bacteria for sure. Altogether the pre and post milking composite samples and the individual quarter samples gave better results: 55.68%, 43.18% and 57.95% respectively. On the bacterium releasing days the results are even better: 72.06%, 55.88% and 75% respectively. The post milking composite samples were cultured three times, and there was a massive raise in the effectiveness, 60.23% (77.94%) was positive, which is almost 20% raise. In the second experiment we found that sometimes only the pre or post milking composite sample was positive while the other was negative. That's why we made a 1 : 1 mixture of these in the third experiment, to see how we can eliminate this problem, but the results were frustrating. The 50% (64.71%) positivity was even less than the result of the pre milking composite samples what is hard to explain. The 1 : 1 mixture of the individual quarter samples, which is actually a post milking composite sample but every quarter gave a same amount of milk into it, gave 52.27% (67.65%) positive results.

Conclusions

After these results we can say, that taking of composite samples before milking is more effective than the post milking ones. It is also clear, that culturing these samples multiple times can raise the effectiveness, just like the costs of these examinations, so before the decision, what to do, we always have to decide how accurate results we need, and is the farmer is willing to pay for the extra costs to get the extra results. Examination of individual quarter samples doesn't raise the effectiveness enough to compensate the four times more costs of labour and tools, so it is not advised in practice unless we need to know exactly which quarter is infected.

New Opportunity of Mycotoxin Elimination

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Summary: It is generally agreed that farm animals should be protected against the adverse effects of mycotoxins. Of the available opportunities, recently the use of microorganisms for biotransformation of mycotoxins has been increasing. Detoxa Plus has been specially developed for enzymatic decomposition of mycotoxins in animal feeds via its active germ component.

The present paper summarise the main findings of three experiments that either had been published *** (Kutasi, J., Papp Z., Jakab, L., Brydl, E., Rafai P.: Deactivation of T-2 toxin in broiler ducks by biotransformation. J. Appl. Poult. Res., 2012. 21. 13 – 20.), being published ** (Rafai P., Papp Z., Jakab l.: Biotransformation of trichothecenes alleviates the negative effects of T-2 toxin in pigs. Acta Vet. Hung. AVH 116/2012) or submitted for publication * (Bata Á, Ványi A., Glávits, R., Brydl E., Rafai P.: Alleviation of the oestrogenic effect of zearalenone in pigs by a feed additive, Acta Vet. Brno, 2013).

Our experiments with growing pigs demonstrated that T-2 toxin impairs feed intake and weight gain of growing pigs at as low as 0. 3 and 0. 5 mg/kg feed concentration. The feed additive used in the present experiment improved the feed intake and daily weight gain of pig significantly. Although parameters of improved feed consumption and weight gain numerically lagged behind those of the control pigs the gap between data of experimentally treated pigs and controls was small and statistically not significant.

Another experiment provided data on the preventive capacity of Detoxa Plus against oestrogen mimetic Zearalenone. The positive effects included the prevention of ovarian and uterine enlargement, beneficial effects on the histopathological scores of the ovaries, uterus and vagina and reduced concentration of Zea and its metabolites in the liver.

In experiments with broiler ducks the adverse effect of 0. 6 ppm dietary T-2 toxin was fully counteracted by the feed additive in terms of daily weight gain, cumulative daily weight gain and live weight at exsanguinations. T-2 toxin depressed the blastogenic response of lymphocytes to non-specific mitogens (ConA and PHA) which was fully prevented by the feed additive.

Introduction

It is generally agreed that farm animals should be protected against the adverse effects of mycotoxins. In principle three ways and their combinations are available for prevention the negative effects of mycotoxins of agricultural importance (e. g. Rafai, 1999). The first approach aims at improving the coping capacity of animals either by extra provision of vitamins and other metabolically active substances, by assisting the immune competence of livestock and poultry via immune enhancing feed additives, or both. Binding mycotoxins in the intestines with surface active materials (e. g. zeolites, glucomannans etc.) represents the second opportunity, although it presents multiple handicaps in terms of binding useful biologically active materials (e. g. vitamins, amino acids etc.) and their limited efficiency against non-polar mycotoxins. A more specific dietary treatment has been recently developed using range of microorganisms for biotransformation of mycotoxins thus rendering them less toxic (Heidler and Schatzmayr, 2003). Of the commercially available products of this last group of mycotoxin detoxifying agents, Detoxa Plus has been specially developed for enzymatic decomposition of mycotoxins in animal feeds via its de-epoxidative effect. The steps of detoxification have been described by Bata and Lásztity (1999). Because of the cell membrane fragments of the active germ the feed additive is claimed to have also mycotoxin adsorptive capacity. Series of experiments were conducted with Detoxa Plus to test the efficiency of the feed additive.

1. Detoxa Plus alleviates the negative effects of T-2 toxin in pigs*

Material and methods

Sixty, 6 weeks old conventional Dutch Landrace × Hungarian Large White F_1 pigs, weaned at 28 ± 2 days of age were allocated on basis of weight and conformation into six groups of 10 and reared for four weeks on a commercially available prestarter and starter feed. Daily feed ration of three groups contained 0 (Group 1, control), 0. 3 and 0. 5 mg/kg T-2 toxin (Group 2 and 4). The feed of another two groups of pigs contained 0. 3

or 0.5 mg/kg T-2 toxin + the feed additive at 2 kg/tonne concentration (Group 3 and 5). One group served positive control; their feed was free from T-2 toxin but contained the feed additive at the same concentration mentioned above (Group 6).

Pigs were kept in flat decks equipped with stainless steel feed troughs, nipple drinkers and aluminium-cast slatted floor at a population density of 5 piglets/pen (0.6 m^2 resting area/pig). Data of the 2 × 5 pigs/group were pooled and processed. Flat decks were housed in climatically controlled chambers at optimum ambient temperature, relative humidity and air velocity. Twelve-hour/day light regime was used.

Weight gain and feed to gain ratio; parameters of energy and protein metabolism; liver function; metabolism of mineral and trace element and parameters of immune function (including antibody formation against purified horse globulin, blastogenic response of lymphocytes challenged with mitogens and phagocytic activity) were investigated.

Results and discussion

Reduced feed intake or complete feed refusal and reduced weight gain are commonly observed when pigs are fed diets with T-2 toxin. In our experiment the average daily feed intake of pigs of either dietary levels of T-2 toxin was consistently and significantly smaller than that of the control group. Supplementation of T-2 toxin contaminated feeds with the feed additive failed to increase the feed intake of experimental pigs to the level of the control pigs, however the difference between controls and pigs of Groups 3 and 5 was substantially decreased by the treatment and the still existent differences were statistically not significant. Supplementation of toxin free diets with the feed additive did not change the overall feed intake.

Both level of T-2 toxin treatment suppressed substantially and significantly the weight gain of pigs in Group 2 and 4 in comparison with the controls. The feed additive alleviated the negative effects of T-2 toxin. In average of the treatment period the weight gain of Group 3 and 5 pigs was numerically inferior to that of the controls but the about 60 g/day difference was statistically not significant. Feed conversion rate of the groups varied between 1.8 and 2.0 kg/kg with no consistent effects attributable to either treatments.

This investigation proved that a dietary concentration of T-2 toxin as low as 0.3 mg/kg can have negative effects on the feed intake and growth of growing pigs. This finding reinforce our earlier conclusion, namely that the no-effect level of dietary T-2 toxin may be below 0.5 mg/kg of feed and supports the suggestion of Eriksen and Pettersson (2004) that lowest limit for sum of dietary T-2 and HT-2 toxin should be set at or below 0.2 mg/kg for pig feeds.

Earlier it was reported (Rafai et al., 1995a) that blood parameters of the energy metabolism was affected only by higher (≥2 mg/kg) dietary level of T-2 toxin. It is therefore not surprising that neither of the parameters tested (glucose; triglycerides, AST; total protein, albumin and urea) showed treatment associated changes in the present experiment. Same applied for parameters of minerals and trace elements.

All pigs built up strong humoral immunity against the purified horse globulin. On day 21 of the experiment the anti-horse globulin titres ($\log_2$) of Group 1 to Group 6 pigs were 4.0 ± 0.55; 4.7 ± 0.35; 4.2 ± 0.28; 4.6 ± 0.32; 4.1 ± 0.48 and 4.6 ± 0.44, respectively. No consistent and no significant differences were found among the titres. In vitro parameters of cellular immunity were comparable to those we reported earlier (Rafai et al., 1995b) and revealed no changes attributable to the treatments.

2. Alleviation of the oestrogenic effect of zearalenone in pigs by Detoxa Plus**

Material and methods

The goal of this study was to test the efficiency of Detoxa Plus in alleviating the presumed negative effects of purified zearalenone (Zea) on growing pigs.

Sixty, 6 weeks old, conventional Dutch Landrace × Hungarian Large White F_1 female pigs weaned at 30 ± 2 days of age were selected from 20 litters and allocated on basis of weight and conformation into six groups of 10 and reared further till 90 days of age on a commercially available starter feed in climatically controlled environment. Between 50 and 70 days of age (experimental period) 4 groups of pigs were treated with purified Zea, dissolved in propylene-glycol at a concentration of 1 mg/ml and administered daily (at 9.00 a.m.) via oesophageal tube either at 8 or 16 mg/pig dose (experimental groups). The remaining two groups was not treated with Zea and served as negative and positive control. These pigs were shame treated with propylene-glycol parallel with the treatment of the experimental pigs. Diet of the two experimental groups and another group of pigs (positive control) was supplemented with the feed additive at 2 kg/tonne concentration. Control pigs received neither Zea nor feed additive treatment. The housing and feeding conditions of pigs were identical with those as described in experiment 1.

Live weight of pigs, weight of ovaries and uterus, histological characteristics of the ovaries, uterus, vagina, spleen and lymph nodes, liver concentrations of Zea and its metabolites (α-and β Zearalenol) were studied to characterise the effects of Zea and alleviating effects of

the feed additive.

Results and discussion

At the time of exsanguination the weight, size and conformation of the pigs were characteristic for the breed, age and sex. Statistical analysis has revealed no significant differences among weight and daily weight gain of groups attributable to the treatments.

After 10 days of treatment with Zeathe ovaries and uterus of pigs treated with the higher dose of Zea had significantly higher weight ($P < 0.05$). The feed additive kept the ovarian weights at the level of the control groups. Higher dose of Zea enhanced the development of the ovarian follicles, had proliferative effects on the uterine glandular layers ($P \leqslant 0.05$) and thickened the stratified epithelium of vagina ($P < 0.05$). The feed supplement reduced the negative effects of Zea ($P \leqslant 0.05$). Further to these the supplementation of the feed with Detoxa Plus significantly reduced the concentration of Zea and its metabolites in the liver of experimentally treated pigs.

3. Deactivation of T-2 toxin in Broiler Ducks by Detoxa Plus***

Material and methods

Effects of two dietary levels (0.6 and 1.0 ppm) of T-2 toxin, and the possible protective capacity of Detoxa Plus were investigated in growing White Pekin ducklings in a 49-day trial comprising six treatment groups of 10 ducks/group. The experimental design consisted of 1 negative and 1 positive control and 4 test groups, as follows: Group 1: negative control, no T-2 toxin and no feed additive added to the feeds; Group 2: positive control, no T-2 toxin added but the feeds were supplemented with Detoxa Plus (2 kg/t); Group 3: the feeds were complemented with 0.6 ppm purified T-2 toxin, no feed additive added; Group 4: 0.6 ppm T-2 toxin + 2 kg/t Detoxa Plus; Group 5: 1 ppm T-2 toxin, no feed additive added; Group 6:1 ppm T-2 toxin + 2 kg/t Detoxa Plus.

Results and discussion

From week 4 till the end of the trial, the daily weight gain of Group 3 ducks was inferior to the controls and the differences of the means were statistically significant ($P \leqslant 0.05$) at weeks 4, and 7, and the final live weight of this group was also significantly lower than that of the control ($P \leqslant 0.001$). The weight gain of ducks in Group 5 was also depressed by the toxin treatment. The adverse effect of 0.6 ppm T-2 toxin was fully counteracted by the feed additive in terms of daily weight gain, cumulative daily weight gain and live weight at exsanguinations. T-2 toxin at both treatment levels depressed the blastogenic response of lymphocytes to non-specific mitogens (ConA and PHA) which was counteracted by the feed additive at the lower dietary concentration of T-2 toxin. No such effect was observed with 1.0 ppm T-2 toxin. Metabolic blood parameters and haematological data showed no consistent treatment effects. It is concludedthat this feed additive can counteract the adverse effects of T-2 toxin at dietary concentrations that might be encountered under field conditions.

Conclusions

Data of the above investigations indicated considerable preventive capacity of Detoxa Plus against a representative of the trychotecens (T-2 toxin) in growing pigs and ducks and also against an oestrogen-mimetic mycotoxin (Zea) in pigs. Because, mode of action of this feed supplement relies more on the enzymatic decomposition of mycotoxins in animal feeds than on adsorbance of toxic materials, the results of the present experiment are very promising.

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Impact of Vitamin E and Selenium Supplementation on Oxidative Stress Indices during Transitional Period of Buffaloe Cows

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Summary: Optimal transition buffaloe health is the key to success in the subsequent lactation. Increasing attention has been focused on management and nutritional practices that support it. The present research, therefore, aimed to investigate the oxidative stress indices in blood during periparturient period and the effects of vitamin E and Se supplementation on them. Oxidative stress was evaluated by measuring steady concentration of free radicals in blood, rate of lipid peroxidation and activity of antioxidant enzymes in erythrocytes, oxidants antioxidant status was evaluated in 22 buffaloe cows. Weekly vitamin E and selenium (Se) supplemented buffaloes (n = 15) was started 8 weeks before calving; the control buffaloes (n = 7) were not supplemented. Blood was sampled 4 times with 2 weeks interval for 8 weeks before calving, on calving day, and weekly done 4 times after calving. Blood samples were analyzed for nitric oxide (NO), Malondialdehyde (MDA), Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GSH-px) and vitamin E. Results showed that concentrations of NO, MDA decreased ($P < 0.001$) in the treated group and tended to increase in the control group. Mean values of antioxidant enzymes activities (SOD, Catalase, GSH-px) decreased gradually before parturition in both groups, after parturition gradual and highly significant increase ($P < 0.001$, $P < 0.01$ and $P < 0.01$ respectively at the 4th week postpartum) was observed in the treated group. Collectively the values of blood antioxidant enzymes activities were significantly higher in treated animals than the control animals during periparturient period. The vitamin E concentration in the blood was greater in the treated group in comparison to control group. Positive correlation was observed between blood MDA and NO, negative correlation was observed between blood MDA, Catalase, SOD, GSH-px and vitamin E. It could be concluded that supplementation of the animal with antioxidants, like vitamin E and Se has beneficial effects on general health condition.

Key words: buffaloe cows, periparturient period, NO, MDA, SOD, CAT, Glutathione peroxidase (GSHPx), vitamin E and Se supplementation

Introduction

Pregnancy and early lactation are stressful stages accompanied with increased metabolic activities and energy demands [7]. The physiological changes during this period result in alteration of normal metabolism and production of stressors that cause diverse disorders [7]. These stages are also accompanied by high energy and oxygen demand which may lead to an increase in the level of oxidative stress and development of metabolic and reproductive disorders in pregnant water buffaloes [10]. [18] Found that the dramatic increase in energy requirements during late pregnancy and early lactation makes dairy cows highly susceptible to negative energy balance (NEB), that occurs in the transition period.

Number of vitamins and trace minerals are involved in the antioxidant defense system and deficiency of any of these nutrients may depress immunity in transition cows. Vitamin E is an important antioxidant that has been shown to play an important role in immunoresponsiveness and health in dairy cows [33]. The present research, therefore aimed to investigate the oxidative stress indices in blood during periparturient period and the effects of vitamin E and selenium supplementation on them.

Material and methods

The experiment was conducted on buffaloe cows related to the Faculty of agriculture, South Valley University. Quena governorate on 22 healthy, lactating, primi or pleuriparous buffaloe cows, 4 – 10 years old. Animals were divided into 2 experimental groups. Group I (Treated group n = 15), buffaloes that received weekly intramuscular injection of 6000 IU α-tocopherol acetate and 67 mg sodium selenite, starting from 8 weeks before parturition and lasted until 4 wk after calving. Group II (Control n = 7) animals served as negative control and did not receive any treatment during periparturient period. Heparinized blood samples were collected. Plasma were separated after and stored at –20℃ till analysis for measuring Nitric oxide [23], Malondialdehyde [27], Catalase [1] and Vitamin E [21]. Remaining packed red blood cells after plasma separation were washed 3 times with physiological saline and resuspended in ice-cold distilled water, shaken vigorously to force haemolysis, and stored at –20 ℃, for measuring Super oxide dismutase [26], Glutathione

peroxidase [28]. The obtained data were statistically analyzed [30].

Results and discussion

The mean blood values of oxidants and antioxidants in treated and control group are shown in Fig. 1 – 6.

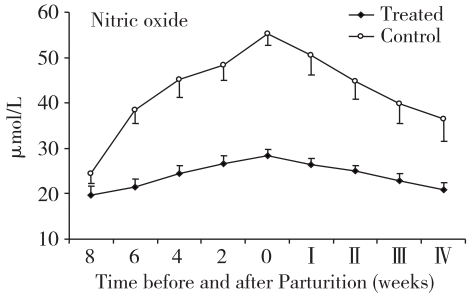

Fig. 1 Mean values (±SE) of blood plasma nitric oxide μmol/L in treated and control buffaloes

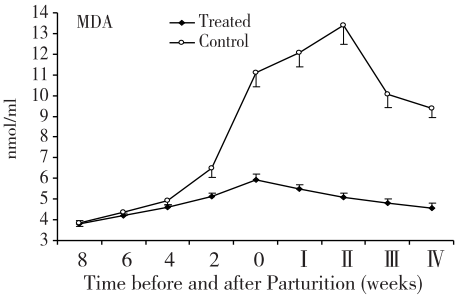

Fig. 2 Mean values (±SE) of blood plasma MDA nmol/ml in treated and control buffaloes

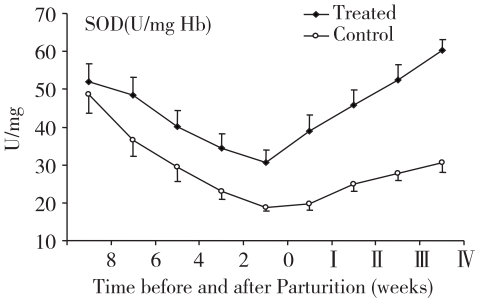

Fig. 3 Mean values (±SE) of erythrocyte lysate SOD U/mg Hb in treated and control buffaloes

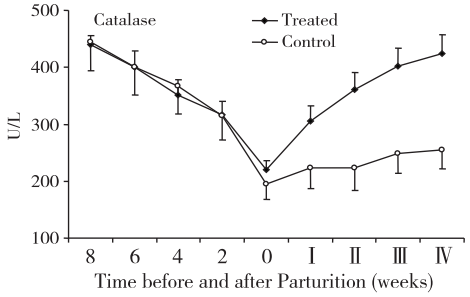

Fig. 4 Mean values (±SE) of blood plasma Catalase U/L in treated and control buffalo

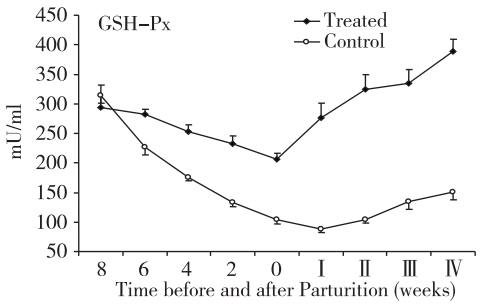

Fig. 5 Mean values (±SE) of blood plasma GSH-px mU/ml in treated and control buffaloes

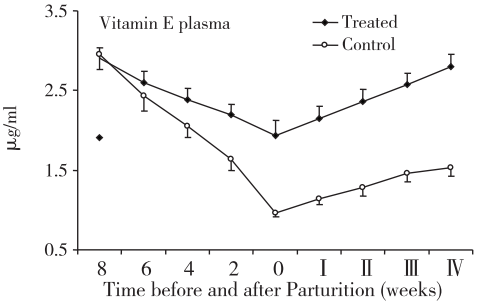

Fig. 6 Mean values (±SE) of blood plasma vitamin E μg/ml in treated and control buffaloes

In studying the relationship between blood and milk oxidative damage and the effect of vitamin E and Se supplementation in buffaloes, we focused on the periparturient period due to the presence of oxidative stress and its possible relation with health problems in the following lactation.

No abnormal clinical signs were detected in the follow up period in all buffaloes in both groups that means animal suffering from oxidative stress may not show any abnormal signs. These findings were in agreement with the finding of [11].

Study indicated that buffaloes were exposed to an increased risk of oxidative stress during the peripartum period, as suggested by the observed increase in NO and MDA concentrations. We noticed that in both groups, NO, MDA concentrations were increased at calving and decrease after parturition. Concentrations of NO, MDA decreased in the treated group and tended to increase in the control group. The lowered MDA blood concentrations of treated buffaloes in the 2 weeks after calving compared with those of control cows suggested that vitamin E has a role in recovering from oxidative stress. The adaptation to a low-energy level involves mobilization of stored energy by breaking down lipids. Lipid mobilization is related to a raised amount of nonesterified fatty acids (NEFA) being taken up by liver tissue to meet energy requirement [8]. During the NEB, NEFA enter into the mitochondria of hepatocytes thus being oxidized to produce energy. Consequently, the increased rate of NEFA oxidation

generates a large amount of reactive oxygen species (ROS) resulting in raised lipoperoxidative processes and changes in the prooxidative/antioxidative status [24]. [4] showed elevated plasma levels of NEFA and ROS around partus. The higher level of lipoperoxidative products, i. e. the MDA concentration in the dry period in our study suggests an exceeding degree of lipid peroxidation caused by ROS in late pregnancy and these findings coincided with those previously obtained by [7]. In the treated group we noticed that vitamin E supplementation could not prevent the increase in blood MDA at calving, but the lower MDA blood concentrations of treated buffaloes in the two weeks after calving if compared with the high level in the control group suggest the role of vitamin E in recovering from parturition-related oxidative stress. These results are in agreement with the findings reported by [5].

In our study SOD, CAT enzymes were decreased gradually from 8th weeks before parturition in both groups and reached the lowest value at the day of parturition. After parturition, gradual and highly significant increase was observed in treated group. Fig. 3, 4 and 5 The observation that ROM levels significantly increased during the peripartum period in dairy cows is in agreement with those previously obtained by [3]. Plasma levels of ROMs are considered an indicator of free radical production [22], and SOD is well known to be a superoxide radicals' scavenger. This is an important factor in the protection against free radical damage [13], and is considered the first defence against pro-oxidants [16]. In the present study also, MDA level did not decrease up to day of parturition even in animals treated with vitamin E and Se, indicating existence of oxidative stress in pregnant buffaloes. Decrease in SOD activity during the pregnancy further consolidated this hypothesis [10]. Furthermore, highly significant increase in SOD, CAT activity on 7th day postpartum in treated buffaloes group indicated the beneficial effects of vitamin E and selenium supplementation during pregnancy. In our study, the higher SOD activity observed in buffaloes on the 4th week postpartum was probably a consequence of lower peroxide generation as testified by the decrease in ROM concentrations observed at the same time. SOD catalyses the dismutation of superoxide to hydrogen peroxide (H_2O_2), and is a key antioxidant defence mechanism in aerobic organisms [16]. Since SOD activity increases H_2O_2 production, protection from reactive oxygen would only be given by a simultaneous increase in Catalase and GSH-Px activities and availability of glutathione [19].

For glutathione peroxidase enzyme we noticed that the mean values of these antioxidant enzymes were decreased gradually from 8 weeks before parturition in both groups and reached the lowest value at the day of parturition in the treated group, while its concentration still decrease till reach the lowest value one week postpartum in control group. After parturition, we noticed gradual and highly significant increase in the mean values of antioxidant enzymes especially in the treated group. Since GSH-Px is directly targeted at removing H_2O_2 generated during the dismutation of free radicals [11], we would have expected a parallel decrease in ROM levels. Reactive oxygen metabolites levels indeed, decreased on the 4th week postpartum; however its concentrations on the 2nd week were significantly high. Even if blood GSH-Px activity was inhibited, the organism could have been defended against oxidative stress by other alternative routes. For example, Catalase is another antioxidant enzyme that can catabolise H_2O_2 [11]. It could be argued that other antioxidant molecules may have played a role in reducing the levels of ROMs during the fourth week postpartum. Administration of vitamin E and Se increases the level of α-tocopherol in plasma, and enhances the biochemical activity of glutathione peroxidase (GSH-Px) [12]. Both are powerful antioxidants and beneficial effects of their supplementation on health and fertility of dairy cows is well established [2]. Therefore, it may be concluded that supplementation of vitamin E along with Se reduced circulating biomarkers of oxidative stress in plasma. Blood GSH-Px was found to decrease during the postpartum period. GSH-Px activity is considered an indicator of oxidative stress [31]. [9] Found that blood GSHPx activity was significantly lower on the 2nd and 4th weeks postpartum, which suggested that the goats may have experienced some degree of oxidative stress and lipid peroxidation.

The finding of increased oxidative stress around parturition [3] and [7], has been substantiated by reduced plasma level of vitamin E during the last month prepartum [20]. In the present study we noticed that the plasma vitamin E level decrease gradually from 8th week prepartum and reached the lowest value at calving day in both groups. Vitamin E concentration in the blood was significantly greater in the treated group ($P < 0.05$) starting from four weeks before calving. In the control group vitamin E concentrations start to decrease in the 6th week before parturition. In both groups, vitamin E concentrations (μg/ml) decreased at calving (control group, $x = 0.96$; treated group, $x = 1.94$) and increased after calving gradually till reach (control group, $x = 1.53$; treated group, $x = 2.80$) one month after calving. Fig. 6.

Vitamin E supplementation resulted in an increase in vitamin E blood concentrations. This is in agreement with other researchs done by [14] and [32]. Supplementation of 3,000 IU/d was almost high enough to prevent a decrease in blood vitamin E concentration prepartum;

other research, using supplementation at 1,000 IU/d, found lower concentrations in prepartum supplemented cows [6] and [29]. [14] Found that the mean plasma vitamin E concentration of 18 cows fell progressively during the last 2 weeks before calving, before increasing by 2 weeks after calving. [17], [15] and [25] found that the mean plasma vitamin E concentrations were higher for the groups given vitamin E supplementation and there was also a significant linear reduction in plasma vitamin E concentration during the dry period, irrespective of vitamin E supplementation.

Conclusions

Finally it could be concluded that Periparturient buffaloes suffer from oxidative stress. Evaluation of the oxidative stress (oxidant status i. e. lipid peroxidation level, enzymatic and non-enzymatic antioxidants) is necessary while conducting studies during periparturient period in ruminants. Vitamin E supplementation reduces oxidative damage in the blood by reducing the levels of both NO and MDA concentrations. In addition providing dry-period buffaloes with vitamin E and selenium supplements (even if they show no signs of clinical illness) would in our opinion help to reduce the risk of oxidative stress.

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Negative Energy Balance in Early Lactation of Cows: Reasons and Risks

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Summary: In dairy cows the high milk yield has lead to a significant reduction of longevity. So-called production diseases such as impaired reproductive performance, lameness, mastitis, metabolic disorders, displacement of the abomasum and increasing mortality rates are the major reasons for early removal of cows from milking herds. The aetiology of these diseases and of the associated mortality is complex, but negative energy balance (NEB) is obviously a predominant cause of production diseases. NEB is a consequence of the homeorhetic regulation of energy metabolism, but the extent and duration of NEB has been increased by selection of high genetic merit cows. Suggestions are made for a better understanding of the causality between NEB and diseases.

Introduction

Dairy cows have been selected over decades for higher milk yield (MY) without a corresponding higher intake of dry matter (DMI) leading to a period of negative energy balance (NEB) [21]. The gap between energy requirement and intake is covered by the mobilization of non-esterified fatty acids (NEFA) from adipose tissue and of amino acids from muscle [20]. Primary selection for high daily MY in the past has had its down-side. High involuntary culling rates are caused by several diseases such as milk fever, ketosis, fatty liver, retained placenta, metritis, mastitis, lameness, decreased fertility, and displaced abomasum [14]. Furthermore, an increase of mortality rates has been observed [1].

The aetiology of diseases and mortality is complex, but NEB is a significant health risk and is linked to ketosis [2], fatty liver [7], reduced fertility [8] and immune suppression [11], lameness [6] and infectious diseases [19].

Hence, the objective of this review is to analyse the mechanisms of homeorhetic regulation of energy metabolism in early lactation, which lead to NEB and NEB related health risks.

Metabolism during transition

The transition period of the dairy cow is characterized by many hormonal and metabolic changes [4, 14]. The term of this adaptation is homeorhesis [4] and characterized by "the orchestrated changes for the priorities of a physiological state, i. e. coordination of metabolism in various tissues to support a physiological state". Many hormones are involved, with a p. p. increase of growth hormone (GH), prolactin, glucagon and cortisol, a decrease of insulin, insulin-like growth factor-1 (IGF-1), tri-iodothyronine and leptin [4, 14]. The major effects of the homeorhetic regulation of energy metabolism p. p. are: a) reduced lipogenesis, b) a decreased uptake of glucose in muscle and adipose tissue, c) significantly enhanced gluconeogenesis in the liver and d) pronounced lipolysis. These alterations of energy metabolism are characteristic for insulin resistance (IR). IR is mainly related to low insulin and IGF-1 levels, the increase of GH and cortisol and a decrease of insulin receptors. Surprisingly, to the best knowledge of the present authors homeorhetic regulation of energy metabolism does not include a *feedback mechanism*, which indeed represents a health risk for cows (see below) because the long-lasting selection for high MY has changed extent and duration of NEB.

Extension of the negative energy balance

a) Initial Milk Yield: MY in early lactation and the ascent to peak yield have a high heritability [12] and have been used for the selection of high MY, which intensified NEB around parturition.

b) Rate of Energy Mobilization: A rapid increase to peak of lactation within 3 – 6 weeks and a daily MY of 30 – 50 kg at peak lactation are considered as "normal" in high-producing cows. The time to peak MY exhibits small variations between high-and low-producing cows [9] and, hence, the acceleration of MY (kg/d) is rapid early p. p. and is significantly higher in high-yielding cows [9, 14].

c) Milk Yield and Dry Matter Intake: The correlation between DMI and MY in early lactation is low [13].

As a consequence of high initial MY, a rapid increase to peak MY and inadequate DMI, the extent of

NEB has changed from < 500 MJ during the eighties of the last century [5] to almost 2000 MJ [18] and the duration of NEB from a few weeks [5] to several months [18]. This change of NEB is directly or indirectly a significant health risk.

Neb as a health risk

Homeorhesis, NEB and Ketosis: BAUMAN and CURRIE (1980) concluded: "Nature has accorded a high priority to the function of pregnancy and milk secretion, allowing them to proceed at the expense of other metabolic processes even to the point that a disease state is created". Hence, homeorhesis includes the risk of a shift from physiological adaptation to pathophysiology as ketosis. BAIRD (1981) stated: "Cows are only susceptible to the disorder (primary ketosis the authors) during early lactation, when *the homeorhetic stimulus to lactate is at a maximum*". During this phase "the cow will attempt to maintain milk production despite food deprivation and as a result will become ketotic"[2].

The possible "disease state" p. p. is the enhanced lipolysis. The rapid increase of NEFA in blood indicates a lipolysis above the actual consumption of NEFA, which are partly metabolized in the liver to ketone bodies such as acetoacetate, acetone and betahydroxybutyrate (BHB) [14]. The resulting hyperketonaemia can lead to subclinical or clinical ketosis. The transition from physiological adaptation to subclinical or clinical ketosis is very likely facilitated by the missing feedback system for control of lipolysis within homeorhesis. NEB is further enhanced by low DMI and ketogenesis is probably augmented by insulin resistance, because insulin is a potent inhibitor of lipolysis. Insulin resistance is an integral part of homeorhesis, which causes release of NEFA. A rapid increase of NEFA is possibly a mediator of (secondary) insulin resistance and triggers further mobilization of NEFA because the damping effect of insulin is missing. Hence, a missing feedback within homeorhesis favours release of NEFA and an impaired feedback mechanism (IR) favours ketogenesis. These two missing control mechanisms could contribute to the high incidence of subclinical ketosis (1.2 – 2.9 $mmol \cdot l^{-1}$ BHB) of > 30% [10, 15]. These phenotypic observations of impaired energy metabolism are in agreement with the known heritability. Heritability of MY, energy balance, BHB and nadir of NEB (maximum of NEB) are high and for DMI low and hence the genetic correlation between MY and ketosis is high [17].

Epidemiological studies have clearly shown that subclinical ketosis around parturition is obviously an indicator of cow performance and of increased risk of diseases later during the lactation period. Hyperketonaemia is associated with displaced abomasum, metritis, clinical ketosis, impaired reproductive performance, early culling and-very important-decreased milk production [10]. Therefore, a significant part of the so-called production diseases are linked to BHB as an indicator of NEB.

NEB and lameness: Cows suffer from lameness with a high incidence and lameness is the second most costly disease in cows after mastitis. A correlation between NEB and the incidence of lameness has been reported for many years, with lameness being most common during the first months of lactation, at time at which NEB is severe. A possible causal link between NEB and the incidence of sole ulcers and white line abscesses has been demonstrated by BICALHO et al. (2009). These claw diseases are significantly associated with the thickness of the digital fat cushion. The mobilization of adipose tissue obviously includes the fat deposition in the digital cushion and follows changes of BCS. BICALHO et al. (2009) have concluded that "these results support the concept that sole ulcers and white line abscesses are related to contusions within the horn capsule and such contusions are a consequence of the lesser capacity of the digital cushion to dampen the pressure exerted by the third phalanx on the soft tissue beneath".

NEB as an unspecific health risk: Beside the direct link between NEB and ketosis and pathogenesis of some diseases of the claw the NEB is an unspecific health risk and related to impaired reproductive performance [8], immune suppression [11], and infectious diseases [19]. However, this interaction is missing a causal interaction and the pathogenesis of the various diseases is not clear.

Conclusion and perspectives

The improved knowledge about regulation of energy metabolism permits some suggestions for further discussion of possible causal correlation between NEB and health risks.

A causal correlation can be deduced between the release of NEFA above requirement and fatty liver, which is a side effect of mobilization and correlated with diseases as displacement of the abomasum, mastitis, metritis and impaired immunreactivity [7]. Hence, fatty liver also exhibits correlations with health risks as NEB and BHB, which clearly shows that a clear cut causal correlation is hardly possible with the current knowledge. With other words, NEB/fatty liver/BHB is a complex health risk and has the same background of an impaired energy metabolism. Therefore clear cut "cause and effect" conclusions are not possible, but unequivocally indicate that all efforts should be directed to minimize NEB.

OLTENACU and BROOM (2010) approached the high incidence of disease in cows from a point of animal welfare and concluded: "At present, considering the severity of the effect on welfare, the duration of the effect and the numbers of individuals involved, after broiler chicken, dairy cows welfare is the worst animal welfare in Europe. Urgent action to change genetic selection and management of dairy cow is needed".

This change could be made easier by the improved knowledge about the regulation of energy metabolism and possible side effects of NEB. A careful analysis of causes and effects within the framework of regulation of energy metabolism should give hints for a better understanding of health disorders and finely for a shift of breeding strategy. Helpful for this discussion could be the well known fact that cows and herds exist with high milk yield and good animal performance. A combination of hypothesis driven research concepts together with modern "omics" technologies should give information both for the reasons that cows are suffering from or cope successfully with the metabolic challenge.

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Black Tea Waste (Bohea Bulu) as Defaunation Agent on Methane Reduction of Rumen Fermentation in Enhancing Friendly Environment of Animal Production

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Summary: This experiment was conducted to determine the effect of black tea waste as a defaunation agent on methane production, during *in vitro* rumen fermentation. Bohea bulu contains 2.95% tannin which can be used as defaunation agent. The treatments in this experiment were tannin levels of 0 (T1), 3 (T2), 6 (T3), and 12 (T4) mg/g dry matter (DM) of feed. A combination of king grass: rice bran (70 : 30) was used as the substrate. Each treatment consisted of three replications. Fermentations, monitored by the gas production technique, were carried out for 72 hours at 39℃. The data were analyzed using ANOVA and the differences of the means were compared by Duncan's new multiple range test (DMRT). The results showed that addition of bohea bulu equal to tannin levels of 3, 6, and 12 mg/g DM of feed decreased protozoa number ($P < 0.05$) by 27.61%, 34.96% and 72.93% and also decreased methane production as well as 27.69%, 62.36% and 70.61% respectively compared to the control. Bohea bulu addition did not affect microbial protein production and pH, but NH_3 concentrations declined ($P < 0.05$) as much as 27.54%, 28.86%, and 39.95% compared to the control. From the results it could be concluded that the addition of Bohea bulu equal to tannin up to 6 mg/g DM of feed could be used to reduce methane production without detrimental affect on the rumen fermentation.

Key words: Bohea bulu, methane gas production, protozoa number, rumen fermentation, *in vitro*

Introduction

Methane is known as one of the pollutants associated with destruction of the ozone. Agriculture is considered to be responsible for about two-thirds of the anthropogenic sources (Moss et al., 2000). Methane from enteric fermentation is a large component of livestock related greenhouse gas emissions, and it has become a special interest for nutritionists. Ruminant nutrition research has been focused on finding methods to reduce methane production, because of inefficiency in ruminant production and not only on the effect on global warming. It has been stated that there is a 6% energy intake loss as methane (Johnson and Johnson, 1995).

Methane is produced as a result of anaerobic fermentation in the rumen and the hind-gut. The rumen methane is produced by methanogenic bacteria and protozoa (O'Mara, 2004). Methanogens, living on and within rumen ciliate protozoa, may be responsible for up to 37% of the total of rumen methane emissions. Strategies for reduce rumen methane emission trough defaunation, removing protozoa population have been reported (Jouany, 1991). This strategies have been applied by additon of tannins, saponins, and essential oil in ruminant feed. Interaction of tannins with protozoa in the rumen is explained by McSweeney et al. (2001) that Tannins cause toxicity to protozoa due to binding to cell walls and impairing cell wall permeability (McLeod, 1974, Makkar, 1993).

Black tea waste (Bohea bulu) is the waste product of black tea processing and is consists of rejected stems and leaves. This waste proportion is as much as 5% to 10% from the total material. The Gambung Tea factories (located in Bandung Region of Indonesia produce black tea, as much as 350 to 960 tons of dry tea per year, and thus produce bohea at an estimated 17.5 to 96 tons/year (PPTK, Gambung, 2006) (unpublished data). The existence of the tannin content in the Bohea bulu has potential to be used as a defaunation agent in the rumen, which is expected to reduce methane emissions, and increase feed efficiency. Bohea bulu as feed ingredient has not been exploited to date, particularly for ruminants. This research objectives are to determine the effect of Bohea bulu addition as a source of tannins on rumen protozoa number and fermentation products including ammonia (NH3), methane gas, and rumen microbial protein production.

Material and methods

Two fistulated rumen of dairy cattle were used to obtain the rumen fluid as a source of rumen microbes for *in vitro* fermentations. The rumen fluid samples were collected early in the morning before feeding and kept at 39℃ aerobically. Bohea bulu, that been used in this research as a source of tannins, were obtained came from the Research Center for Tea and Quinine, Gambung

factories, Bandung, West Java, Indonesia.

In vitro rumen fermentation has been done by *in vitro* gas production technique. Fermentation was conducted in the syringe as a fermentor. 200 mg of feed material, consisted of King grass and rice bran (in ratio 70 : 30 DM basis,) was put into the syringe and followed by addition of Bohea bulu equal to 0 (T1), 3 (T2), 6 (T3), and 12 (T4) mg/g of feed tannin level, and then incubated overnight at 39℃. Mixture of rumen fluid and buffer (1 : 2), was then pumped in 30 ml doses into syringes. Each treatment consisted of three replicates. Gas production over 72 hours incubation was recorded in hours at 2, 4, 6, 8, 12, 36, 48, and 72 hours. Gas sample were taken at the end of incubation as much as 10 ml from each syringe and collected separatly in vacum tube for futher methane analysis by Gas Chromatography (GC). Fluid samples from each syringes were collected for protozoa number according the method of Diaz et al. (1993), and fermentation products determinatin. The data were analyzed using ANOVA design and the All multiple comparisons among means' was performed using Duncan's new multiple range test (DMRT) (Astuti, 1981).

Results and discussion

The addition of Bohea bulu decreased the protozoa number ($P < 0.05$) compared to the control (without Bohea bulu addition) as much as 27.61%, 34.96% and 72.93% respectively for addition Bohea bulu equal to 3, 6 and 12 mg/g of feed tannins level (Table 1). Similar to data of on the protozoa, methane production also decreased by the addition of Bohea bulu ($P < 0.05$) (Table 1). The decrease of methane production, respectively for T2, T3, and T4 was 27.69%, 62.36% and 70.61% (Table 1).

Table 1 Protozoa number, gas production, methane production and characteristics of rumen fermentation at several level of Bohea bulu addition as the tannin source.

Parameter observations	Tannin levels (mg/g DM of feed)			
	0	3	6	12
Protozoa number ($\times 10^3$/ml)	15.29[a]	11.06[b]	9.94[b]	4.22[c]
Gas production (ml/300 mg BK)	66.26[a]	50.52[b]	39.77[c]	33.41[c]
CH_4 production (ml/200 mg BK)	4.57[a]	3.31[b]	1.72[c]	1.35[c]
Characteristics of rumen fermentation microbe				
pH	6.96[a]	6.89[ab]	6.87[ab]	6.82[b]
NH_3 Concentration (mg/100 ml)	41.78[a]	30.28[b]	29.73[b]	25.09[b]
Microbe protein (mg/100 ml)[ns]	53.52	42.56	40.92	42.23

[abc] The values in the same row with different superscripts show the different effects of treatment ($P < 0.05$).
[ns] not significant.

The pH did not affected by treatment on T2 and T3 but with T4 the pH was decreased by the Bohea bulu addition ($P < 0.05$). The addition of Bohea bulu on rumen fermentation *in vitro* decreased the ammonia concentration ($P < 0.05$), whereas microbial protein was not influenced (Table 1). The mechanism of the decrease of the protozoa number because tannin has a toxic effect on the protozoa by binding to the cell walls and impairing cell wall permeability (McLeod, 1974, Makkar, 1993). In the rumen, the formation of methane is the major pathway of the hydrogen sink through the folllowing reaction: $4H_2 + CO_2 \longrightarrow CH_4 + 2H_2O$ (Takahashi, 2006). The decrease in methane gas in this experiment was caused by the decline in protozoa number as an effect of the tannin in the bohea. H_2 is one of the major end products of fermentation activity of protozoa, fungi and pure monocultures of some bacteria (Moss et al., 2000). The collaboration between fermenting species, particularly protozoa and H_2 utilizing bacteria (e.g. methanogens), is called "interspecies hydrogen transfer" (Jordan et al., 2006). Several techniques to remove protozoa from rumen have been conducted experimentally, but none of them have been applied routinely, due to toxic problems either to the rest of the rumen microbial population or to the host animal. Recently, interest in exploiting plant secondary metabolites for defaunating agent has increased. In particular, tannin-containing plants show promise as possible means of suppressing or eliminating protozoa in the rumen, without inhibiting bacterial activity (Moss et al., 2000).

At the end of the fermentation, the pH of all four treatments were still in the normal range. The normal rumen pH is 6.3 to 7 (Ørskov, 1992). These results are also consistent with the work of Santoso (2006) where in vitro *B. petersianum*, which contains 1.1% tannin, did not influence pH. The NH_3 concentration decreased by the addition of Bohea bulu. Subrata (2005) reported that there were effects of tea on soya bean meal fermentations reduced significantly the levels of ammonia from 5.62 mM to 1.24 mM. Meissner et al. (1993) stated that fermentation of feed containing tannins in the rumen produced ammonia with lower concentration than feed that does not contain tannins. The decrease in ammonia

concentrations was due to the addition of Bohea bulu, as tannins reduce protein degradation in rumen through a tannin-protein complex, which protects the protein from attack by microorganisms (Carulla et al., 2005).

The addition Bohea bulu, equal to 0 (T1), 3 (T2), 6 (T3), and 12 (T4) mg tannins/ml of mix feed on *in vitro* rumen fermentation, had no effect ($P > 0.05$) on microbial protein. McDonald et al. (2002) explain that the NH_3 concentration in the rumen ranges from 85 to 300 mg/l, and as further described by Orskov (1992), no increase in microbial protein synthesis occurs as a result of increasing ammonia concentrations in the rumen to more than 50 mg/l. The results showed that a decline in the levels of NH_3 did not affect microbial rumen protein synthesis, because the levels of NH_3 in the medium were still in the normal range for growth of rumen microbes.

The main factor for microbial protein synthesis is the availability and concentration of precursors in the rumen i.e., carbon chains, NH_3, energy sources (ATP) and sufficient minerals (Ørskov, 1992). Jouany (1991) reported that the ciliate protozoa contributed significantly to intraruminal cycling of microbial N and to the efficiency microbial protein, thus reducing the protozoa counts can improve dietary N utilization and increase the microbial protein flow to the intestine (Makkar, 1998).

Conclusions

Bohea bulu is a potential material that can be used to develop an environmentally sound livestock process by decreasing methane emissions. Addition of Bohea bulu equal to a tannin level up to 6 mg/ml medium did not affect the rumen fermentation.

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Bacteriological Assessment of Feed and Intestinal Microbial Characterization of Pigs Fed on Garbage

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Abstract: In India, pig production systems are mainly based on low cost agro-industrial by-products. Kitchen waste is a cheap source of feed. Feeding kitchen waste to pigs has positive economic and environmental implications. Bacterial contamination of food is the most frequent cause of food borne disease. Cooking of waste food to prevent transmission of diseases may also minimized this risk. An experiment was conducted on 27 crossbred weaned pigs aged 2 – 2. 5 months. The pigs were separated into three groups consisting of nine piglets in each group in a completely randomized design and they were subjected to three different feeding treatments (ad. lib.) viz., T1 Concentrate mixture, T2 Raw kitchen waste and T3 Boiled/cooked kitchen waste. The pigs were reared up to 5 months of age. A total of 360 samples including 180 faecal samples from three groups of crossbred pigs and 180 samples of three types of diet viz. concentrate mixture, raw kitchen waste and boiled kitchen waste were collected and processed. Significantly ($P < 0.01$) highest body weight gain was observed for T2 followed by T3 and T1. The highest percentage of prevalence of bacteria ($P < 0.01$) was found in raw kitchen waste and faecal samples of pigs of group T2. The prevalence of *E. coli* was found higher than other enteric bacteria faecal samples of pigs. Prevalence of *Staphylococcus* spp. and *Clostridial* spp. were isolated mostly from raw kitchen waste. Cooking or boiling of kitchen waste significantly reduces the bacterial count ($P < 0.01$). Bacteriological quality was observed best in boiled kitchen waste followed by concentrate and raw kitchen waste. In conclusion, raw kitchen waste must be processed before feeding to pigs so that these unacceptable practices do not constitute persisting environment, animal and human health hazard.

Intestinal Immunity of *Escherichia coli* Nissle 1917: Safe Vehicles for Targeted Therapeutics

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Abstract: The probiotics *E. coli* Nissle 1917 has been widely used in medicine and cattle production industry. *Escherichia coli* strain Nissle 1917 is a non-pathogenic fecal isolate, combining both the excellent colonization properties and its non-immunogenic character, and can be administrated orally. Genetically modified probiotics can very well be an ideal candidate as carrier organism for gut-focused in situ synthesis and expression of specific localized antigen delivery into the intestine. And for the first time, a live genetically modified bacterial strain has been approved by Dutch authorities as a therapeutic agent for experimental therapy of intestinal bowel disease (IBD) in humans. Therapeutic safety, however, of such a carrier organism for targeted therapy, is crucial, especially when a specific probiotic strain has to be used under diseased conditions where the barrier function of the epithelial layer could be easily destroyed. In the light of studies, it demonstrate that intestinal recombinant *E. coli* Nissle-HA110-120 has the potential to stimulate antigen specific response *in vitro*, but does not induce mucosal immune response and influence peripheral tolerance to self-antigen. Furthermore, recombinant *E. coli* Nissle 1917 has no effect on migration, clonal expansion and activation status of specific CD4 + T cells, neither in healthy mice nor in other animals with acute colitis. After that, a series of studies have shown that *E. coli* Nissle 1917 can be a tumor-targeted delivery of TAT-Apoptin fusion gene to colorectal cancer. Besides, it has a great significance in the development of mucosal vaccine, especially for orally application, and also important for the recombinant strain used as a carrier for target presenting defensins in the treatment of ulcerative colitis and Crohn's disease.

Effect of using Nano Elemental Selenium on Immunity in Poultry Nutrition

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Abstract: The main object of this work was to investigate the effect of adding nano elemental selenium (Nano-Se), Selenium yeast (Sel-Plex) and Sodium Selenite (NaSe) to the diets of chickens on chickens immunity and performance . The experimental design consisted of six experimental groups: control and 5 dietary treatments as follows; (T1) Basal diet (control), (T2) Basal diet + Sodium Selenite (NaSe) (3. 0 ml/kg diet), (T3) Basal diet + Selenium yeast (Sel-Plex (3. 0 ml/kg diet), (T4) Basal diet + Nano-Se (0. 1 ml/kg diet), (T5) Basal diet + Nano-Se (0. 3 ml/kg diet) and (T6) Basal diet + Nano-Se (0. 5 mg/kg). Characteristics investigations included : Immune response (immune response against NBVD and Cell-mediated immunity (Cutaneous basophil hypersensitivity CBH). Results obtained can be summarized as following: It is clearly evidenced that feed additives significantly improve the natural immunity of birds against viral invasions. An average antibody titer recorded in control diets was always significantly less than those found in all feed additives treatment (T2 to T6 diets). Also, The addition of Nano-Se with different levels, Sel-Plex and NaSe to the chicks diet had significant increased in CMI response as compared with control , although in many cases NaSe seemed to be less effective than others feed additives. In addition , Nano-Se (0. 3 ml/kg diet) was more affected than other levels of Nano-Se.

Dietary Supplementation with Different Butyrate Species in Growing Rabbits

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Abstract: The aim of the study was an assessment of an influence of sodium butyrate (unprotected and protected) and calcium butyrate in growing rabbits feeding. An influence of additives used on body weight gains, health and carbohydrate-lipids transformations in blood and short-chain fatty acids profile was examined. The experiment was performed on 48 rabbits of genetic meat line (Hyplus) divided onto four equal groups: control group, group receiving unprotected sodium butyrate, group receiving protected sodium butyrate and rabbits receiving unprotected calcium butyrate. Control diet was without butyrate addition, while particular additives were used in an amount of 1% of dry matter of complete fodder. The preparations were applied on fodder using spraying method. The rabbits were given experimental fodder from 17th to 60th day of life. The weaning took place in 42nd day of life. Blood analysis were performed in 42nd and 60th day of life, and glucose, cholesterol and triglycerides were determined. Also markers of liver function (GOT, GPT, GGT and total bilirubin) were examined. In the 60th day of life rabbits were subjected to euthanasia and total VFA concentration as well as acids from C2 to C5 were determined in caecum content using chromatography method. The highest body weight gains were noted in rabbits supplemented with protected sodium butyrate, however the differences were not confirmed statistically. Sodium butyrate (unprotected) contributed to a significant increase in glucose concentration in blood ($P < 0.05$). Long-term application of butyrate in an unprotected form may be related to liver functions disorders, what is proved by an increase in liver enzymes activity. The additives applied did not influence concentration of C2 – C4 fatty acids in caecum of rabbits. Protected sodium butyrate caused a significant ($P < 0.05$) increase in total VFA concentration.

Environmental Pollution, Emissions and Abatement Options

Bee Products as Potencial Bioindicators of Heavy Metal Contamination

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Summary: The aim of the study was to determine the bioaccumulation level of Zn, Cu, Pb, Ni and Cd in honey and bee pollen collected from two regions characterized by different degrees of environmental contamination. The samples of each apiary products were collected from stationary apiaries located in industrialized (IN) and non-industrialized (N-IN) areas of Southwest Poland. Quantitative analysis was performed by using Varian ICP-AES plasma spectrometer with controlled mass detection, and CETAC-5000 AT ultrasonic nebulizer. The results showed higher level of all examined heavy metals in both types of bee products in samples from industrialized area in comparison with non-industrialized region. In bee pollen and honey samples from IN area zinc showed the highest concentration: 36. 95 $mg \cdot kg^{-1}$ and 4. 58 $mg \cdot kg^{-1}$, respectively. Whereas the lowest level was determined in case of cadmium: pollen 0. 56 $mg \cdot kg^{-1}$ and honey 0. 14 $mg \cdot kg^{-1}$. The same tendency was observed in samples from NIN region (bee pollen: Zn 33. 63 $mg \cdot kg^{-1}$, Cd 0. 41 $mg \cdot kg^{-1}$; honey: Zn 4. 85 $mg \cdot kg^{-1}$, Cd 0. 09 $mg \cdot kg^{-1}$). All heavy metals showed higher concentration in pollen samples in comparison with honey samples. The results of the presented research lead to conclusion that both subjected bee products could be used as bioindicators. It seems however that bee pollen is better bioindicator than honey in respect to environmental contamination.

Introduction

Recent development in industry and motorization together with intensive agriculture based on the chemicalization, have contributed to a massive increase of environmental pollution. Among many pollutants there are elements of toxic properties, whose natural content in soil and the atmosphere is insignificant [2, 5, 14]. Nevertheless, as they are widely used in the human economy, they are common environmental pollutants. Even their low concentrations may cause vascular diseases, kidney or bones damages, irregular functioning of human and animal reproductive system. They can easily penetrate to the cell membranes and internal organs as well as cause denaturation of proteins in blood or mucous membranes and penetrate to the tissues. Honey bees (*Apis mellifera* L.) are 100% dependent on the flowering plants which provide them with nectar and pollen. The pollutants occurring within the area where bees are working can be accumulated in their organisms and also in the raw material collected by them [9, 11, 12]. Therefore, bees and bee products such as honey and bee pollen can be a valuable indicator material in the investigation of environmental contamination. There is a close correlation between the level of heavy metals accumulation in soil and plants and their content in bee products [1, 3, 13].

Material and methods

The research material constituted samples of multiflower honey and bee pollen obtained in the form of pollen loads. The samples of each apiary produce were collected from stationary apiaries located in industrialized (IN) and non-industrialized (Non-IN) areas of Southwest Poland. The IN area consisted of agricultural-woodland regions such as Kłodzko Valley and Lubsza commune. Second study area (Non-IN) included the Legnica-Glogow Copper District (LGOM) which is main center of copper industry in Poland and it is one of the major center of the world's copper operation.

Material was collected from May to July 2011. Each of both studied products were collected from 3 bee colonies in 35 apiaries. The individual samples were combined into one pooled sample weighting about 50 g, representative for each apiary. Honey was collected directly from honey combs by cutting off part of the cells and squeezing honey. Bee pollen loads were collected in traps located at the entrance of hives. The pollen trap brushed the pollen off the pollen baskets of foraging worker bees as they were entering the hive.

The received samples were homogenized as follows: honey-by carefully mixing; bee pollen loads-by drying, fragmentation and mixing. The 1000 mg of material from each sample was weighted (with precision of 0. 1 mg) and diluted with 20 ml of concentrated, spectrally pure, nitric acid solution produced by Merck company. Next,

samples were mineralized using the microwave technique at an elevated pressure in the chip-type MD-2000 station manufactured by CEM-USA. Quantitative analysis was performed by using Varian ICP-AES plasma spectrometer with controlled mass detection, and CETAC-5000 AT ultrasonic nebulizer. All analyses were conducted in Analytical Laboratory of Wroclaw University of Environmental and Life Science (Poland). The results of the research were elaborated statistically by ANOVA. The mean concentrations of elements, standard deviations and correlations between elements were calculated. Significant level was taken as $P \leqslant 0.01$.

Results and discussion

The present study demonstrated that bee pollen samples exhibited higher level of toxic elements contamination in comparison with honey samples in both researched regions. The Zn concentration was the highest in pollen samples from IN region. Its average value was 36.95 $mg \cdot kg^{-1}$. In the case of N-IN area, Zn content was 33.63 $mg \cdot kg^{-1}$. The level of this metal in honey samples from IN and N-IN area amounted to 4.58 and 4.85 $mg \cdot kg^{-1}$, respectively. The concentration of zinc in pollen was many fold higher than in case of honey. Comparable low level of zinc in honey samples was noted by Forte et al. [7] and Stankovska et al. [14]. Also very similar results were observed by Tuzen et al. [15] and Yazgan et al. [16]. However, Caroli et al. [4] found that the quantities of zinc in honey were considerably higher than those obtained in the present study. In case of zinc contamination in pollen samples, similar results were noted by Harmanescu et al. [8] in pollen samples coming from Romania. The Cu content in pollen samples from IN and N-IN area was 10.03 and 7.55 $mg \cdot kg^{-1}$, respectively. Whereas in honey samples these values were lower and amounted to 6.42 and 3.89 $mg \cdot kg^{-1}$, respectively. The concentration of Cu in pollen samples from N-IN region was significantly higher in comparison with honey. In comparison with present study lower copper concentration in honey was obtained by Forte et al. [7] and Stankovska et al. [14]. In turn, Pisani et al. [10] observed copper concentration lower than 0.100 $mg \cdot kg^{-1}$. Another heavy metal whose concentration was higher in bee pollen in comparison with honey was Pb. Its average concentration in samples from IN and N-IN region amounted to 5.14 and 4.15 $mg \cdot kg^{-1}$ respectively. Whereas in honey the presence of lead was at an average level of 1.56 $mg \cdot kg^{-1}$ for IN and 1.33 $mg \cdot kg^{-1}$ for N-IN region. Statistically significant differences ($P \leqslant 0.01$) between the level of Pb in both bee products from different regions were observed. Very low content of this metal in honey from Rome suburbs was also evaluated by Conti and Botrè[6]. Similarly, small amounts of lead in honey from Turkey were observed by Tuzen et al. [15]. In case of bee pollen average concentration of lead in samples from both studied areas was higher than that reported by Conti and Botrè[6]. Generally, the smallest concentration in the examined bee products was observed for cadmium. As in case of most of the previous trace elements, the highest concentration of cadmium was noted in bee pollen from IN region, where its average level was 0.56 $mg \cdot kg^{-1}$. Lower level of these elements was observed in case of honey samples from N-IN area and averaged 0.09 $mg \cdot kg^{-1}$. Statistically significant differences ($P \leqslant 0.01$) between the level of Cd in both bee products from different regions were observed. Considerably lower cadmium content in honey originating from Turkey was obtained by Tuzen et al. [15]. Also very low concentrations of cadmium were obtained by Stankovska et al. [14]. In case of bee pollen average concentration of lead in samples from both studied areas was higher than that reported by Conti and Botrè[6]. The Ni content in pollen samples from IN and N-IN area was 2.49 and 3.05 $mg \cdot kg^{-1}$, respectively. Whereas in honey samples these values were lower and amounted to 1.10 and 0.88 $mg \cdot kg^{-1}$, respectively. Considering the nickel concentration in the tested bee products, it must be noted that there are statistically significant differences between bee products coming from different regions. The nickel level in our honey samples was higher than that reported by Caroli et. al [4] near the subjected regions.

Conclusions

The results of the present research allow to conclude unequivocally that the level of toxic trace elements concentration in bee pollen and honey depends on the state of environmental pollution in the area of the material sampling. Therefore, both subjected bee products could be used as bioindicators. The results however showed that bee pollen is better bioindicator than honey in respect of environmental contamination.

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Evaluation of Heavy Metal Level in Sheep and Goat Exposed to Lead Toxicity in Bagega, Zamfara State Nigeria

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Summary: Lead is a common environmental and occupational contaminant widely distributed around the world. In 2010, a high level of lead contamination was recorded in Bagega district of Zamfara state, Nigeria due to the wide spread local mining activities. This lead poisoning epidemic in northern Nigeria (Zamfara state) is the worst in modern history and has killed about 400 children and affected thousands. Humans are not alone in suffering from the effects of lead contaminated environment as animals are also affected to varying degrees due to their widespread husbandry in the area. The system of sheep and goat husbandry in this area equally expose the animals.

Whilst there is growing concern over the potential detrimental impact of lead toxicity in environment, little is known about their interactions with other contaminants. In the present study, the epidemiology (prevalence) of blood lead level (BLL) and other associated heavy metals in small ruminants of the affected areas and environs were evaluated.

This study showed varying levels of BLL and other associated heavy metals like, cadmium (Cd), chromium (Cr), manganese (Mn), Zinc (Zn) and selenium (Se). This data from animals are good indicators of environmental contamination and also a measure of food contamination from polluted livestock in the food chain.

Introduction

Heavy metals constitute a serious cause of medical concern to both human and animals. This is due to its very toxic effect and its tendency to bioaccumulate and be recycled into the environment (Hart et al., 2005; Merrill et al., 2007). Human activities generate tonnes of such toxic environmental contaminants. Some of these environmental contaminants are the heavy metals which also constitute a major natural component of the environment (Reena et al., 2011; Nor et al., 2012). Lead contamination from mines have been reported to coexist with other heavy metals (Alma et al., 2000). These heavy metals such as cadmium, lead, mercury, chromium and copper constitute a serious environmental and health problem with lead being the most toxic of them followed by cadmium (Humphreys, 1991; Patra et al., 2011). The levels of these heavy metals despite their ubiquitous presence is often increased and spread over a wider distance by human activities such as mining and industrial processes. This is typified by the high level of lead contamination as reported by Medecins San Frontieres (Doctors without Border MSF) in 2010, in Zamfara State, due to illegal artisanal gold mining activities prevalent in the area (UNICEF final report, 2011). This lead poisoning epidemic has been described by the Human Right watch as the worst in modern history and has killed over 400 children and affected thousands. Humans are not alone in suffering from the effects of lead contaminated environment as animals are also affected to varying degrees (Merrill et al., 2007).

Ruminant livestock production in Nigeria is predominantly carried out in the Northern region of Nigeria of which Zamfara is a part. This poses a nationwide public health risk to the whole country that depends on livestock from this lead polluted part of the region.

In the present study, the epidemiology (prevalence) of blood lead level (BLL) and other associated heavy metals in small ruminants of the affected areas and environs were evaluated to assess their absolute values and the level of coexistences with other heavy metals.

Material and methods

Animals

50 samples of the 250 samples collected from small ruminant (sheep and goat) in Bagega, a village in Anka local government area of Zamfara where the lead toxicity in humans were reported, were analysed for this preliminary study.

Samples

Blood samples (5 ml) were collected from the jugular vein in vacutainer tube containing heparin, transported to the laboratory and kept in a freezer (−20℃) for analysis.

Blood digestion and determination of heavy metal concentration

Whole blood was digested with Concentrated Nitric acid (HNO_3). 1 ml of whole blood was measured into clean test tubes; 1 ml of HNO_3 containing 0.1% triton-100 was added and allowed to mix thoroughly. On the

second day, the mixture was then heated in a water bath at 100℃ for 20 min, thereafter allowed to cool. The digested blood samples were transferred to a measuring cylinder and the volume made up to 25 ml with distilled water. Lead and some heavy metal concentration in the digested blood were measured with BULK SCIENTIFIC, model 205 Atomic Absorption Spectrophotometer AAS/ Atomic Emission Spectrophotometer.

Statistical analysis

All data was expressed as Means was done using SPSS Statistical Tool 17.0.1, 2008 software.

Results and discussion

The average blood lead level was 31.94 μg/dl. 90% of the animals had detectable blood lead levels, while 78% had levels above the tolerable level of 5 μg/dl. 96% of the samples had detectable cadmium levels which were above the tolerable levels. There were detectable levels of other heavy metals as shown in table 1 below.

Table 1 Showing the distribution of heavy metal in blood

Metal	No. of samples	Mean value detected (μg/dl)	% of samples with detectable levels	% of samples exceeding tolerable levels	Highest value detected(μg/dl)	Tolerable Blood Levels (μg/dl)
Pb	50	31.94	90	78	417.37	0 – 108*
Cd	50	19.76	96	96	45.9	0.02 – 0.5**
Cr	50	10.05	62	64	154.6	0.05 – 0.2**
Cu	50	37.97	100	100	53.8	7.5 – 15*
Se	50	23.38	100	90	44.9	9 – 16*

* Centre for Disease Control CDC

** Agency for Toxic Substances and Disease Registry ATSDR

In relation with the results obtained from the human studies (Talha, 2012), this study shows values which confirms that the animals are equally in contact with the lead contaminated environment and also shows levels of toxicity. The result also show that other heavy metals were present and in toxic levels which indicates that lead is not the only heavy metal contaminant. Therefore the reported incidence, morbidity and mortality could be due to combined heavy metal toxicity and needs further studies. The result gives an insight to the situation and calls for further research using the small ruminants as indicator animals.

Conclusion

There was a presence of toxic level of lead in the tested population, this was however also the case with other heavy metals. There is therefore the need for further research into the interactions of heavy metals in the biological system.

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Residues of Chronic Rodenticides in Plants after Their Application to Soil

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Summary: The study focused on observation of residues of chronic rodenticides KUMATOX G with active ingredient warfarin, BRODER G with active ingredient brodifacoum and DERATION G with active ingredient bromadiolon in relation to their residues in wheat grown on rodenticide-treated soil. The preparations were applied to soil in the form of granular baits. The results obtained showed that residues of the respective rodenticides were present in the crops.

Introduction

The recent reports show frequent infestation of field crops with pests. The most often encountered pests are the field vole (*Microtus arvalis*) and common hamster (*Cricetus cricetus*). Due to the damages caused by these rodents, the farmers may decide to control them using rat control preparations. According to the list of preparations registered for protection of plants and other preparations in Slovakia, issued by the Ministry of agriculture of SR in 2006, the preparations based on brodifacoum could be applied previously in open spaces; at the present zinc phosphide is used for this purpose. It was observed that some farmers, when short of money, may occasionally decide to reduce costs by applying cheaper pest control preparations or preparations they have in store. Up to the present, few studies paid attention to potential occurrence of rodenticide residues in the relevant crops in relation to the fact that individual plants take nutrients from soil treated with rodenticides.

These considerations motivated us to investigate the potential absorption of rodenticides by field crops and, in the positive case, also presence of residues in the relevant crops.

Material and methods

The toxic baits were applied at the beginning of March at sowing of grains (wheat) in the amount of 100 g/m^2 and 500 g/m^2 of bait. The preparation KUMATOX G containeds first generation rodenticide warfarin in 0.025% concentration, which corresponded to 2.5 g/m^2 and 12.5 g/m^2 of warfarin in the bait, respectively. Preparations BRODER G and DERATION G contained rodenticides of second generation, brodifacoum and bromadiolon, in concentration of 0.005% which came up to 0.5 g/m^2 and 2.5 g/m^2 (5 kg/ha and 25 kg/ha), resp., in the applied bait. The bait was applied onto the surface of soil (soil group regosols, soil type regozem) placed in containers of dimensions 1 m × 1 m × 0.5 m and worked into the soil. No other agrotechnical measures were taken throughout the vegetation period. In early August, at harvest time, the wheat was scythed manually and respective samples (whole spikes) were collected. Control samples were obtained from wheat grown on untreated soil.

Determination of rodenticide residues

The samples collected were thoroughly homogenized and 10 g aliquots were extracted for 15 min with 25 ml of mixture of chloroform + acetone (1∶1). The solvent was decanted through a filter paper and the extraction was repeated once more with fresh solvent.

The extracts were combined and the solvent was evaporated at approx. 40℃ in a rotating vacuum evaporator. The residuum was dissolved in 1 ml methanol and 1 ml of mobile phase was added to remove co-extracted proteins. The solution was then transferred into a centrifugation tube containing n-hexane and centrifuged for 10 min at 3500 r. p. m. The cleared lower layer was filtered and used for HPLC chromatographic analysis.

HPLC chromatography

The chromatographic analysis was carried out on a column LiChrospher^R 100 RP-18 (5 μm) with mobile phase acetonitrile (A) + acetate buffer, pH 4.6 (B), (50 + 50) for warfarin, (60 + 40) for bromadiolon and brodifacoum, at a flow rate of 1 ml · min^{-1}, injected aliquot 10 μl and UV detection at 265 and 310 nm.

Results

Our study focused on determination of rodenticide residues in wheat grown in the soil treated with the respective rodenticides.

After application of 100 g/m^2 Kumatox G to soil, the

residues in wheat ranged between 0.034 and 0.044 ppm and the dose of 500 g/m^2 resulted in residues ranging between 0.051 and 0.065 ppm.

After application of 100 g/m^2 of the rodenticide bait Broder the residues in wheat were in the range 0.012 – 0.022 ppm and the dose of 500 g/m^2 reselted in residual levels between 0.034 and 0.044 ppm. The 100 g/m^2 dose of Deration bait resulted in residual level 0.012 – 0.018 ppm and that of 500 g/m^2 in residual level 0.03 – 0.043 ppm.

In comparison with human ADI (acceptable daily intake) for brodifacoum (0.0000005 mg·kg^{-1} day), after applying the dose of 100 g·m^{-2} soil, the ADI for 60 kg man (0.00003 mg/kg/day) was exceeded 400 – 733 times and with the dose of rodenticide bait equal to 500 g·m^{-2} soil the ADI was exceeded 1133 – 1466 times. When comparing the human ADI set for bromadiolon (0.000002 mg·kg^{-1}·day^{-1}), after applying the dose of 100 g·m^{-2} soil, the ADI for 60 kg man (0.00012 mg/kg/day) was exceeded 100 – 150 times and with the dose of rodenticide bait equal to 500 g·m^{-2} soil the ADI was exceeded 250 – 358 times.

Discussion

Recently considerable attention has been paid to the quality of the environment and its contamination with various chemical pollutants, for example radioactive substances (Dvořák et al., 2006) and heavy metals (Sasáková et al., 2006). This is related to increased frequency of various civilisation diseases for which it was discovered that their primary cause was chemicals released to the environment.

Warfarin was the first anticoagulant rodenticide developed in the period from 1947 to 1948 and registered in the USA in 1952 (Thompson, 1991). It is a part of various commercial rodenticides and presently is also used in human medicine, indicated in conditions associated with a risk of production of thrombi. The extensive use of warfarin in practice increases the risk of its residues in the environment. There is no information available on warfarin residues in soil or ground or surface waters. This was the reason why we investigated occurrence of warfarin residues in soil and plants grown on warfarin-treated soil. We detected residues of warfarin in soil even three years after its application which may be related to its chemical properties, such as its insolubility in water (OHS DATABASE, 1994, Windholtz 1983), melting point 159 – 165℃ (Farm Chemical Handbook, 1994) or 318 – 322 °F (OHS DATABASE, 1994) and solubility in acetone, chloroform and dioxans (Montogomery, 1993, Hayes, 1963). The properties mentioned above suggested that it can persist in soil for a long time after application and its elimination may be very difficult. Some authors described its decomposition by soil micro-organisms (Kunc, 1974), for example *Streptomyces griseum* (Sima Sariaslani, Rosazza, 1983). With regard to its insolubility in water we consider the presence of its residues in crops surprising. No natural synthesis of warfarin was described in cereals in contrast to other types of plants, such as *Mikania glomerata*, Sweet clover (Melilotus alba and M. officinalis) (Renata et al. 2001). Information is not available regarding the ability of cereals to absorb warfarin by their metabolism or possibility of simple adherence of warfarin to plant surface during the growth. Occurrence of warfarin residues in plants rises a question of its low dose, long-term action on animal and human organisms.

According to the current legislative provisions rodenticide concentrates are included in the group of extra-hazardous poisons which, however, does no apply to commercial rodenticide baits. Due to their high toxicity to animals considerable attention has been paid recently to their effects on non-target species. Booth et al. (2001) described acute toxicity of brodifacoum to invertebrates, such as snails, and to terrestrial species of crabs. Eason et al. (2001) investigated residues of brodifacoum in poisoned wild pigs and goats and Booth et al (2003) observed toxic action of brodifacoum on earthwarms. All the studies pointed to the need of monitoring and evaluation of the effect of rodenticide anticoagulants on invertebrates and development of residues in plants after application of rodenticides in nature. Of the relevant chemical properties of brodifacoum we should mention its insolubility in water, melting point ranging between 228 and 230℃ and its solubility in acetone and chloroform (Hayes, 1963). These properties allow one to assume that its residues may persist in soil for a long time and remediation of soil contaminated with brodifacoum may be difficult.

In our study we investigated the occurrence of residues of brodifacoum, the active ingredient of the second generation rodenticide BRODER G, with 0.005% concentration of brodifacoum in the bait. Brodifacoum is a bromylate derivative of hydroxycoumarin which was used for the first time in 1975 to control rodents resistant to coumarin. Our results indicated unambiguously the risk of residual levels of brodifacoum in plants grown on rodenticide-treated soil. The residual levels correlelated with the dose of BRODER G applied to the soil

The solubility of bromadiolon in water is lower than 20 mg·l^{-1}, its melting point is in the range 200 – 210℃ and it is soluble in acetone and chloroform (Hayes, 1963). This resembles the properties of brodifacoum and similar risk of persistence of residues in the soil and potentially in the crops. Bromadiolon is an active ingredient of the second generation rodenticide

DERATION G and the content of bromadiolon in the bait is 0.005%. Bromadiolon is a bromylate derivative of hydroxycoumarin, used for the first time in 1976 to control rodents resistant to coumarin. Our examination for the residues of bromadiolon in wheat showed persistence of this substance in crops grown on bromadiolon-treated soil, dependent on the applied dose of DERATION G.

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Exploring Novel Therapeutics for *Cryptosporidium*: Identification and *in vitro* Screening of Phylomer® Peptides and Chinese Herbs

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Summary: *Cryptosporidium* is a protozoan pathogen that causes prolonged diarrhea in humans, livestock, wild animals, birds, fish and reptiles. The parasite can be transmitted via the faecal-oral route and by contaminated water. The only approved treatment in humans, nitazoxanide (NTZ), is ineffective in immunocompromised individuals and new anti-cryptosporidial therapeutic agents are urgently required. *Cryptosporidium* lacks de novo purine synthesis, and is exclusively dependant on purine salvage from its host. Inhibition of inosine 5' monophosphate dehydrogenase (IMPDH), a purine salvage enzyme that is essential for DNA synthesis, offers a potential drug target against this parasite. A previous study used a yeast-two-hybrid system to identify phylomer peptides (constructed from the genomes of phylogenetically diverse bacteria) that targeted the IMPDH of *C. parvum* (IMPDHcp). Here we present dost response analysis of two phylomers, which exhibited significant growth inhibition (81.2% – 83.8% inhibition; $P < 0.05$), when screened against *C. parvum* in vitro. We also present data on the anticryptosporidial activity and cytotoxicity of 7 traditional Chinese medicines (TCMs) *in vitro*.

Introduction

Cryptosporidium is an important gastrointestinal, zoonotic waterborne pathogen and the most common non-viral cause of diarrhoea in humans and animals. Cryptosporidiosis can result in severe diarrhoea, dehydration, abdominal pain and weight loss. These symptoms are usually limited to two-week duration, however in patients who are immuno-compromised, the disease can be persistent and chronic, often becoming life threatening (Carey et al., 2004). Currently, nitazoxanide (NTZ) is approved for treatment of cryptosporidiosis in children and immunocompetent adults in the U. S. A., however NTZ is not effective without an appropriate immune response and is therefore ineffective for the treatment of immunocompromised individuals (Gargala 2008).

Like other apicomplexan parasites, *Cryptosporidium* is unable to synthesize purine nucleotides de novo and relies exclusively on inosine monophosphate dehydrogenase (IMPDH) for purine salvage (Abrahamsen et al., 2004). The IMPDH gene appears to have been acquired through lateral gene transfer from an ε-proteobacterium and is very different from its human homologs (Striepen et al., 2004). The "drugability" of IMPDH is well established as inhibitors of human IMPDHs have been used clinically as immunosuppressants as well as for the treatment of viral infections and cancer (Chen et al., 2007; Hedstrom, 2009). Thus, the exclusive reliance on the salvage pathway by *Cryptosporidium* and its high metabolic demand for nucleotides due to the parasites complicated lifecycle make IMPDH a potential drug target candidate.

A previous study identified phylomer peptides (which are highly diverse naturally stable protein segments derived from bacterial genomes) that interacted with *C. parvum* IMPDH using yeast forward-two hybrid assays (Jefferies et al., 2013). Here we present the results of dose response assays for two of these phylomers against *Cryptosporidium* using an established means of delivery of peptides across the *Cryptosporidium* cell membrane (the HIV-1 derived TAT protein transduction domain (PTD)) (Chin-Lee et al., 2008) and an in vitro culture system that supports the entire *Cryptosporidium* life cycle (Hijjawi et al., 2002).

We also present data on the anti-cryptoporidial activity of Traditional Chinese Medicines (TCM's), which are rapidly gaining attention in the West as sources of new drugs, dietary supplements and functional foods.

Material and methods

Phylomer® preparation and *in vitro* screening

A total of 13 phylomers, which had previously been shown to interact with *C. parvum* IMPDH were synthesised as TAT-fusion proteins as previously described (Jefferies et al., 2013). The phylomers were dissolved in 100 μL of 80% DMSO in water to obtain a primary stock concentration of 8 mM. The Phylomer® - DMSO solution was incubated at room temperature for 30 min, then vortexed to encourage dissolving. Aliquots

were stored at – 20℃. *Cryptosporidium* was grown on human ileocecal adenocarcinoma cell line (HCT-8) host cells (ATCC 244) as previously described (Hijjawi et al., 2002; Jefferies et al., 2013).

Phylomers® were introduced into cultures at 24 hr post infection at concentrations of 80 μM, 16 μM, 3.2 μM, 0.64 μM and 0.128 μM. Positive controls were HCT-8 cells infected with oocysts, while negative controls were HCT-8 cells with heat killed oocysts and uninfected HCT-8 cells. Five μL of 80% DMSO was added to positive controls to ensure that DMSO at <1% did not inhibit HCT-8 cell growth. Duplicate TAT peptide controls were included in each screen.

Each screening plate also included two positive drug controls; Trifluralin (TF) (0.1 μM) which has previously been shown to produce 50% – 70% inhibition of *C. parvum* growth *in vitro* at that concentration (Armstrong, unpublished data) and mycophenolic acid (MPA), which is a weak inhibitor of IMPDH (10 μM and 50 μM) (Abrahamson, 2004). Screen plates were incubated for 48 h in 5% CO_2 at 37℃. After 48 hours, maintenance medium was removed from wells by aspiration and discarded. The cell monolayers were washed once in sterile 1 × PBS and the DNA was extracted and the plates screened by quantitative PCR (qPCR) as previously described (Yang et al., 2009; Jefferies et al., 2013).

TCM screening

Seven TCMs were purchased from a Chinese medicine shop in Perth, Western Australia: The herbs were dissolved in water at 4 mg/ml. The concentrations of TCMs tested were 0.4 mg/ml, 0.8 mg/ml and 1.2 mg/ml, respectively. The *in vitro* anti-*Cryptosporidium* screening was conducted as described above.

Measurement of cytotoxicity of phylomers and TCMs

Cytotoxicity to host cells was determined using an *in vitro* toxicology assay kit, Tox7 (Castle Hill NSW, Australia), which measures lactate dehydrogenase as a guide to cell integrity. The assay was performed according to the manufacturer's instructions and both positive and negative controls were used. The assay was performed in duplicate.

Guanine recovery assay

As IMPDH targets guanine salvage, a guanine recovery assay was performed to determine if addition of guanine resulted in reversal of inhibition. Cultures were incubated with Phylomer® 8 at 6.4 μM, 3.25 μM, 0.64 μM and 0.128 μM, respectively for 48 hours and then 50 μM guanine was added to each well, a further 200 μl cell growth media was added and the plates were incubated at 37℃ for another 48 hours. At this point, the wells were washed and the DNAs were extracted and inhibition analysed by qPCR.

Results and discussion

***In vitro* screening of Phylomers®**

Of the 12 phylomers® tested, two phylomers® (phylomer 8 and phylomer 24) exhibited significant levels of inhibition (Fig. 1). Phylomer 8 was a peptide derived from *Haemophilus influenzae* and Phylomer 24 was derived from *Chlorobium tepidum*. Both phylomers exhibited 81.2% – 83.8% inhibition respectively against *Cryptosporidium in vitro* at the highest concentration tested (80 μM). The control drug MPA inhibited parasite growth by 45.64% at 50 μM ($P < 0.0053$). Trifluralin inhibited parasite growth by 53.13%. Cytotoxicity analysis revealed no toxic effects of the phylomers on the cells.

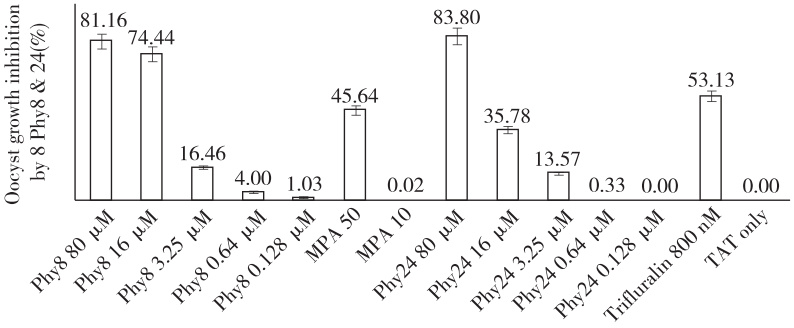

Fig. 1 Effect of Phylomer 8 and Phylomer 24 on *C. parvum* growth inhibition *in vitro* at 80 μM, 16 μM, 3.2 μM, 0.64 μM and 0.128 μM concentrations. The error bars represent the 95% confidence intervals of parasite growth reduction. MPA was tested at 10 μM and 50 μM. Trifuralin was tested at 0.1 μM.

Guanine recovery assay

An inverse relationship was observed between the phylomer concentration and guanine recovery i. e. the lowest concentration of phylomer tested (0.128 mM) resulted in the highest recovery with 50 μM guanine (86.2%) (Table 1). The highest concentration tested

(6.4 mM) resulted in 41.3% recovery. The no guanine treated controls exhibited no decrease in inhibition. This result suggests that the phylomer was inhibiting IMPDH, as the addition of guanine resulted in a reversal of inhibition.

Table 1 Guanine recovery efficiency assay tested on phylomer 8

Phylomer concentrations	No Guanine Inhibition rate(%)	50 μM Guanine inhibition rate(%)	Recovery rate(%)
TAT only control	0	0	–
0.128 mM	6	0.8	86.2
0.64 mM	29.4	13.2	55
3.25 mM	51	24	53
6.4 mM	80	47.3	41.3

The present study further supports previous research, which identified IMPDH as a good drug target in *Cryptosporidium* (Sharling et al., 2010) by identifying phylomer peptides that interact with and inhibit IMPDH resulting in inhibition of *Cryptosporidium* growth *in vitro*. In recognition of the fact that peptide-based anti-parasitic drugs are unlikely to be either affordable or commercially viable in developing-world settings, future studies will focus on the development of small molecular compounds that specifically disrupt the activity of IMPDH.

Anti-cryptosporidial activity of TCMs

All 7 TCMS exhibited various levels of *C. parvum* growth inhibition (Fig. 2). H7 (Sophora Flavescents Ait) exhibited the highest inhibition (100%), followed by H5 (Herba Andrographitis Ait) (97.8%), H2 (Berberine) (96.2%), H1 (Herba Artemisiae) (76%), H6 (Garlic extract) (75.2%), H3 (Herba Houttuyniae) (64%) and H4 (Herba Andrographitis) (69%), at the highest concentration tested (1.2 mg/ml). However, H7 and H2 also exhibited with the highest cytotoxicity rates (16.7% and 12.5% respectively) at that concentration. H1, H3, H4 and H5 had lower cytotoxicity levels at 1.2 mg/ml concentration (7.3%, 6.4%, 5.9% and 9.3% respectively) (Fig. 2) and should be evaluated further using a large dose response range with pure compounds extracted from herbs.

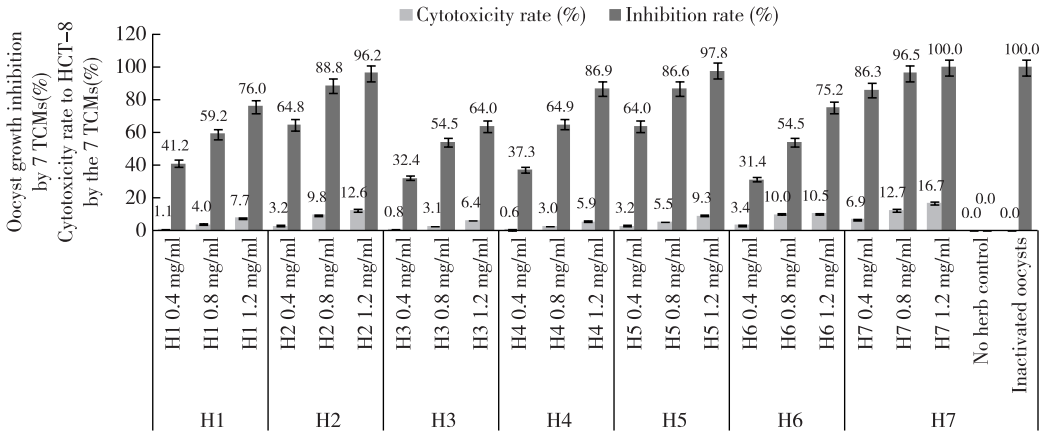

Fig. 2 Effect of 7 TCMs on *C. parvum* growth inhibition and their cytotoxicity on HCT-8 *in vitro* at 3 concentrations (0.4 mg/ml, 0.8 mg/ml and 1.2 mg/ml). The error bars represent the 95% confidence intervals of parasite growth reduction or the HCT-8 cytotoxicity rate.

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Effects of Acidification on Ammonia Emissions from Pig Digested Slurry

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Summary: Reduction effects of acidification with different pH value adjustments on ammonia (NH_3) emissions from digested slurry storage were investigated. Three parallel pilot-scale experiments were conducted including two different acidified treatments compared to the control group with non-treatment in this study. Pig digested slurry was stored in specially designed reactors. The pH values and temperatures in digested slurry were continuously monitored by sensors. Ammonia emissions from the 9 reactors were regularly sampled by atmospheric sampling instrument and then determined byspectrophotometer. Ammonia emissions were high for non-treated digested slurry during storage. The reduction efficiency on NH_3 emissions was 44.7% for treated group with pH value adjusted to 5.5, while no significant difference was found in treated group with pH value acidified to 6.5 compared to control group.
Key words: acidification; digested slurry; ammonia; reduction effects

Introduction

Livestock production has been reported to be a crucial contributor of NH_3 emissions into the atmosphere. Ammonia can be emitted from animal housing, manure management and subsequent land applying [1]. Ammonia volatilization not only lowered the nitrogen content in organic fertilizer, but also was a public concern for its negative impact on the environment.

Knowledge about NH_3 releases from livestock manure composting and slurry storage has been investigated in previous field work. Gas production is a complex process results from microbial and chemical reactions [2]. The amount of gas emissions mainly depend on manure characteristic, management approaches and climate condition [3-4]. Releases of gases also vary related to manure types [5] and feeding operations. The pH value also has been proved to be an important factor to affect NH_3 emissions, and several technologies and approaches have been reported in the literature for reducing NH_3 emissions from livestock manure by lowering pH value [6-8].

As an effective and environment-protecting utilizing method of livestock manure, anaerobic digestion technology has been paid much attention. In china, more and more CAFOs apply for biogas engineering system to treat animal manure and wastewater. But problems arise when considering the utilizing of digested slurry, which is rich in nutrients and has both high economic and environment value, can't be used directly and must be stored for few months. Only a few studies related to the behavior of gas releases from digested slurry storage were found in literature [9-11]. Study of gas releases from digested slurry and abatement technology is still not enough, so more knowledge is needed regarding gases emissions and how different covers and other handling technology affect the reduction of gas emissions during digested slurry storage.

The overall objective of this research was to study NH_3 releases during digested slurry storage and investigate the effect of acidification with different pH adjustment on NH_3 emissions.

Material and methods

Experimental setup

The study was conducted in Room D708, College of Biosystem Engineering and Food Science, Zijingang Campus, Zhejiang University. The setup was designed to avoid odorous gases from the digested slurry in the lab. Nine reactors were used to store digested slurry. Each reactor consisted of a small container and a large one (both were made of PE). The large container was 74 cm tall and 64 cm in diameter, and the small one 64 cm × 50 cm. The total volumes of the two containers were 150 L and 100 L respectively. The small container was open and was placed in the large container that was sealed with an air tight cap. There was one port installed on one side of the center in the cap for fixing the temperature and pH probe. The air inlet port was installed on the wall of the big container 10 cm above the bottom. Another port was installed in the opposite side about 5 cm below the top for gas sampling to determine NH_3 concentration and exhausting the extra gases outside the lab.

Digested slurry was collected from a commercial swine farm in Zhejiang Province. The fermentation process was about 5 days before the fresh digested slurry

were discharged from biogas digesters. The fresh digested slurry was sampled and instantly transported to the lab for chemical properties analysis. The initial volume of the digested slurry in each reactor was 75 L. The whole experiment lasted for 95 days. To evaluate the reduction effects on NH_3 emissions during the storage through lowering down the initial pH value, the three digested slurry groups before the storage were treated differently as follows: (1) digested slurry with no treatment (control group); (2) digested slurry acidified to pH 6.5 using sulfuric acid (treated group 1); (3) digested slurry acidified to pH 5.5 using sulfuric acid (treated group 2). Each group was conducted in triple for repetition.

The reactors were left undisturbed during the storage period except during sampling for NH_3 and determining pH values using a pH electrode when the probes didn't work. The fresh ventilation air was continuously supplied by an air compressor. The airflow rate in all the reactors was regulated to 6.2 – 6.7 $L \cdot min^{-1}$ through needle-value and gas flow meter. Sampling for NH_3 was conducted every 3 days in the first 52 days and every 5 days when NH_3 concentration was relatively low.

Measurements

Digested slurry were analyzed for total nitrogen (TN), total phosphorus (TP), chemical oxygen demand (COD), ammonia nitrogen (NH_4^+-N), total sold (TS) and Volatile (VS) according to the standard of the Ministry of Agricultural of the people's Republic of China both at the beginning and the end of the experiment. TS was determined after drying samples at 105 ℃ for 24 h. VS was measured after burning the samples at 600 ℃ in muffle for 2 h. TN was determined by spectrophotometer after the samples were digested by alkaline potassium persulfate. TP was determined by ammonia molybdate spectrophotometric method. NH_4^+-N of samples was analyzed by the colorimetric method. COD was measured by potassium dichromate method. Ammonia emission was sampled for 10 minutes at a flow rate of 1L/min by atmospheric sampling instrument (Laoying 2020, Laoshan, China) and then determined by spectrophotometer.

Although manure pH values at different depths have proved to be different in previous study [2], only pH values in the middle height of the digested slurry was selected in this experiment. Temperatures in the room and digested slurry were measured at the same location of the pH probes (XW-300, Luheng, China. The theory percentage slope ⩾ 95%) using temperature sensors (YL01, Yulai, China. The deviation is 0.12 ℃). Both the pH probes and temperatures were calibrated before usage. When the pH probes didn't work, a pH electrode (PB-10/21, Sartorius, Germany) was used to measure the pH values at intervals.

Inlet air flow rates were adjusted using a dry gas flow meter (Defender 510H/520H, Bios International Corporation, USA. The precision was ± 1%). Its measurement range was 0.3 – 30 L/min. All the dates were collected by AIRDAC system developed by Ni et al. (2011) [12] from Purdue University.

Statistical analysis

Ammonia concentration and pH values were analyzed by analysis of variance using SPSS software (version 20, International Business Machine Corp) and no significant difference was found at the 0.05 significance level for the three repetitions in each treatment. LSD test was followed when samples were determined to be statistically different through comparing the results between different treatments. Pearson coefficient was acquired by correlation analysis using SPSS software. The reduction efficiencies (%) in methane emissions by different pH adjustments were calculated according to Eq.

Reduction efficiency = 100 – *Relative concentration*

Where the *relative concentration* (%) was defined as *concentration above treated slurry/concentration above untreated slurry*) × 100

Results and discussion

Digested slurry characteristics

The initial and final digested slurry analyses are given in Table 1.

Table 1 Digested slurry characteristics

	Start of experiment	End of experiment		
		Control	Treated 1	Treated 2
Volume (L)	75	72.17	72.19	72.25
TS ($g \cdot L^{-1}$)	1.55	3.5	4.2	7.1
VS($g \cdot L^{-1}$)	0.87	1.36	1.48	3.67
Ash ($g \cdot mL^{-1}$)	0.68	2.18	2.72	3.41
TN ($mg \cdot L^{-1}$)	1.467	705	718	949.4
TP ($mg \cdot L^{-1}$)	162.8	51.74	55.10	72.86
COD ($mg \cdot L^{-1}$)	3.777	1.331	1.900	2.378
NH_4-N ($mg \cdot L^{-1}$)	987.3	630	638.3	879.9
pH	7.46	8.55	8.0	7.95

Values are arithmetic means from two repetitions per treatment, and values from two repetitions have no significant difference at the 0.05 probability level.

Values of pH and temperatures

The mean pH values over the total investigation period were shown in Fig. 1. For the control group, the initial pH value was 7.5 and almost kept steady till day 10, then gradually increased to 8.4 on day 38. The finding was in line with Huang et al. (2012) [13]. For the treatment groups, the pH values increased sharply from 5.5 to 7.20 in the first 25 days and from 6.5 to 7.75 in the first 20 days respectively. This may be

explained by the changes of microbial activities in digested slurry after acidification. For the treatment with pH adjustment to 5.5, there was a significant increase of the pH value from day 54 to 55.

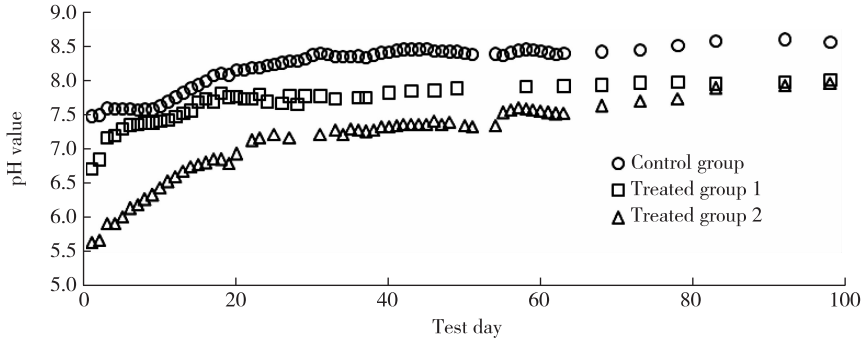

Fig. 1 Courses of the pH values during digested slurry storage. The incoherency of the date markers was due to the pH probes damage.

As shown in Fig. 2, the room temperature fluctuated from 22 to 35℃. The variability of the temperatures in the digested slurry corresponded with that of the room environment, and the overall trend was lowering down during the whole storage due to the natural climate change.

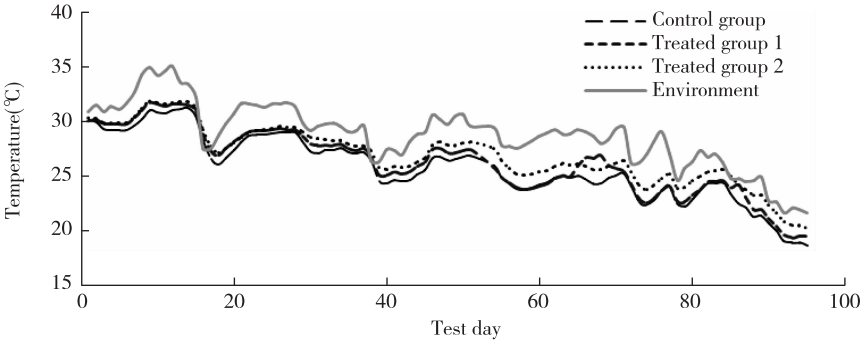

Fig. 2 Temperatures of the digested slurry during storage

Ammonia emissions

There was a strong positive correlation between NH_3 emissions and temperature ($r = 0.785$), and negative correlation between NH_3 emissions and pH value ($r = 0.814$), indicating that NH_3 concentration changes during the storage could attributed to both the two factors.

Ammonia emissions from treated and non-treated digested slurry during storage are shown in Fig. 3. No predictable NH_3 emissions changes were found in control group. Ammonia emissions from digested slurry in treated group 2 were significantly lower than the control group and treated group 1. While acidifying digested slurry to 6.5 didn't show obvious potential to reduce NH_3 emissions compared to control group. The sulfuric acid added lowed pH value of digested slurry and changed the balance of NH_3 and NH_4^+ in the solution, which promoted NH_4^+ production and simultaneously inhibited ammonia volatilization. So at the beginning after acidification to pH 5.5, NH_3 emission was significantly mitigated and kept steady at low level during the whole storage. After day 43, the temperature increased which accounted for the increasing of NH_3 in treated group 2. In treated group 1, NH_3 emissions were inhibited in the first three days like treated group 2, then began to sharply increase from 19 mg/m^3 on day 3 to 71 mg/m^3 on day 12. From day 12 to day 22, NH_3 concentration in treated group 1 kept high and even exceeded control group. Then decreased to 21 mg/m^3 on day 28 and kept stable during the following storing period.

There was significant difference ($P < 0.05$) between treated group 2 and control group, suggesting that acidification with initial pH adjustment to 5.5 reduced NH_3 emissions effectively, especially in the first 52 days. Previous studies reported acidifing livestock maure with pH value below 6 could reduce NH_3 emissions by 67% [6], and 77% with pH adjusted to 5.5 [14]. While in this study, the daily mean reduction efficiency for NH_3 with pH adjusted to 5.5 through acidification was only

44.7%, and the maximum reduction efficiency was 83.7% which occurred on day 6. Ammonia emissions on day 6, day 21, day 43 and day 72 in three groups were given in Fig. 4.

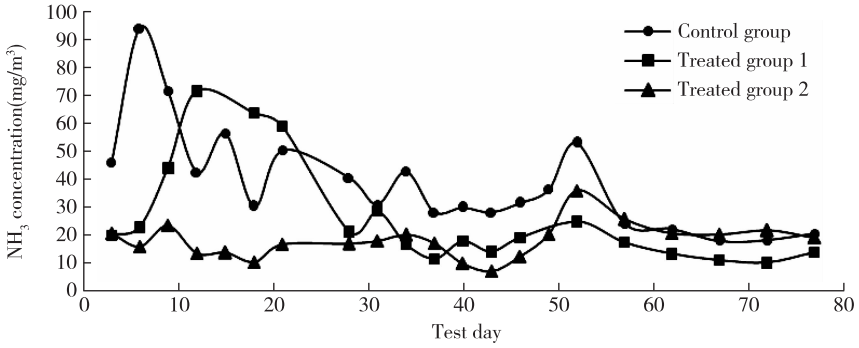

Fig. 3 Daily mean NH_3 concentration during digested slurry storage

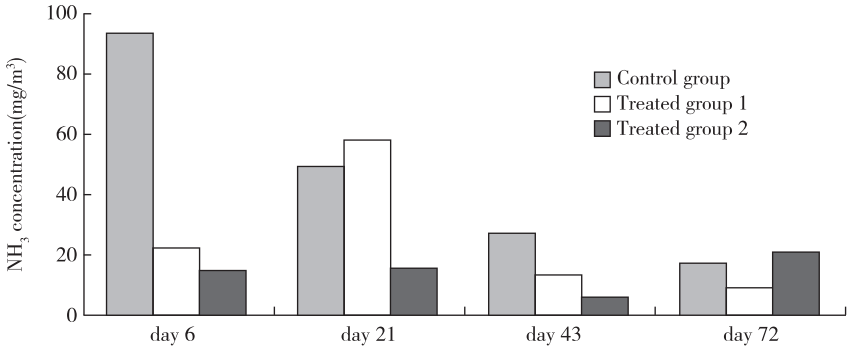

Fig. 4 Daily mean NH_3 concentration on test day 6, day 21, day 43 and day 72 respectively

Conclusions

Temperature and pH value are both crucial factors to influence NH_3 emissions during digested slurry storage. Ammonia emissions were high for non-treated digested slurry during storage. Using sulfuric acid to adjust pH value of digested slurry to 5.5 could reduce NH_3 efficiently and the daily mean reduction efficiency was 44.7% compared to control group. No significant difference was found between treated group 1 with pH value adjusted to 6.5.

Acknowledgement

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Emissions of Odorogenous Pollutants in the Process of Disposal of Dead Animals

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Abstract: The aim of the study was to assess the level of air pollution by odorous compounds in the process of disposal of dead animals. Air samples for chromatographic analysis were taken in the area of a meat waste processing plant and in its vicinity. The studies revealed the presence of air sulfur compounds of a markedly odorogenic nature, such as hydrogen sulfide, sulfides, disulfides and merkaptans. The average concentration of sulfur compounds (including unidentified ones) in the air of the hen house reached 15.4 – 389.6 $\mu g/m^3$. The highest concentrations of diethyl sulfide and methylopropyl sulphide were observed in ventilation channel.

Ecotoxic Effects of a Veterinary Food Additive, $CuSO_4$ on Antioxidant Enzymes and mRNA Expressions in Earthworms

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Abstract: This study was aimed to investigate the effect of veterinary food additive $CuSO_4$ ($CuSO_4$) at different concentrations and different early exposure phases, on the ecotoxic responses of the earthworms *Eisenia fetida* (*E. fetida*). Earthworms were exposed to increasing concentrations of $CuSO_4$ (50, 100, 200 and 400 mg/kg dry soil by Cu) and for different periods of time (5, 10 and 15 days). The following biomarkers were measured: Catalase (CAT), superoxide dismutase (SOD), glutathione peroxides (GPx), glutathione-S-transferase (GST) activity and malonaldehyde (MDA) concentration. Moreover, gene expression analysis was also conducted as molecular biomarkers: the expression of metallothioneins (MT) gene and heat shock protein 70 (Hsp70) gene. These biomarker responses varied in relation to concentrations and exposure times. The results showed antioxidant enzymes had the efficiency to provide antioxidant defenses against the stressor except GST and highlighted expression of MT and Hsp70 could be used as reliable and sensitive molecular tools with predictive possibilities to monitor the ecotoxicity of veterinary feed additive $CuSO_4$.

Key words: veterinary food additive; antioxidant; expression; $CuSO_4$; ecotoxicity

Evaluation of HSP70 and Antioxidant Enzymes in *Eisenia fetida* as Early Warning System to Roxarsone and Arsanilic Acid Ecotoxicity

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Abstract: Roxarsone and arsanilic acid are exhaustively used as the animal and poultry feed additives around the world, breaking down to release arsenic, are a matter of concern for the scientists. So, during this study full length sequence of heat shock protein (HSP70) was achieved from *Eisenia fetida* and succefully established as biomarker to assess the eco-toxicity of roxarsone and arsanilic acid. HSP70 has the size of 2481 bp encoding 655 amino acids, 72. 99 kDa molecular mass and exhibited significant similarity to HSP70 of other species. QRT-PCR revealed the significant differential gene expression in response to roxarsone and arsanilic acid treatment as compared to control treatment. Roxarsone caused the highest gene expression 59. 34 folds to control on 15th day of treatment against the concentration 150 mg arsenic/kg of soil. On the other hand, arsanilic acid initially resulted in the suppression of the gene expression but reached to the highest 44. 35 folds increase to control on final day of treatment against the concentration 300 mg of arsenic/kg of soil. Classic oxidative biomarkers SOD, CAT and GSH-PX played their role as well by fluctuating in response to the roxarsone and arsanilic treatments at different time intervals. The highest SOD (43. 12%), CAT (64. 56%) and GSH-PX (30. 50%) activity was found on day 10 (SOD) and day 15 (CAT &GSH-PX) against roxarsone (SOD) and arsanilic acid (CAT &GSH-PX) respectively. In general all the concentrations of roxarsone and arsanilic acid caused the fluctuations in HSP70 gene expression and oxidative biomarkers to some extent at different time intervals. These findings not only establish the HSP70 as a potential upcoming molecular biomarker but integrate it with the traditional oxidative biomarkers.

Effect of Some Disinfectants on Elimination of *Salmonella* spp. from Post-fermentation Liquid

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Abstract: Co-fermentation of slurry makes it possible to obtain alternative energy in the form of biogas, manage waste organic substances and generate valuable fertilizer. However, in the case of failure or improper course of process parameters, liquid and sludge left after fermentation may pose a sanitary hazard.

The aim of this study was to examine the effect of selected disinfectants on the elimination of bacilli of the genus *Salmonella* from post-fermentation liquid deriving from an agricultural biogas plant.

Material for the study was post-fermentation liquid from an agricultural biogas plant constituting a waste after mesophylic methane co-fermentation of pig slurry. The liquid was contaminated with bacilli of *Salmonella*, whose number amounted to 10^3 MPN × cm^{-3}. Post fermentation liquid was poured to glass vessels 1 dm^3 to each. One of them was the control, and the studied compounds were introduced to the others, obtaining the following final concentrations: soda lye – 1.0% and 1.5%, perhydrol – 2.0%; 3.0% and 4.0%, burnt lime – 1.0%; 3.0% and 5.0%, hydrated lime – 1.0%; 3.0% and 5.0%, waste soda lime (20% – 30% CaO) – 50, 100, 150, 200 and 300 kg/t. After mixing 10 cm^3 of material was collected for the study and placed for 15 min in PBS pH = 7.2. Then the number of *Salmonella* bacilli was determined with the MPN method using culture media usually applied in microbiology. The interval of sample collection was 5 min, 1 hour and 1, 2 and 4 days.

The most effective disinfectants turned out to be soda lye and perhydrol, which caused the complete elimination of *Salmonella* bacilli as early as after 5 minutes in each of the applied concentrations. Applying burnt and hydrated lime in a concentration of 3% and 5% the complete inactivation of the studied bacteria was observed after 1 day and 1 hour, respectively. The rate of those substances providing a concentration of 1%, in turn, eliminated bacilli of *Salmonella* during 2 days in the case of CaO, and after the use of $Ca(OH)_2$ the number of those microorganisms still remained on a level of 10^1 MPN × cm^{-3} on 4^{th} day. Using waste soda lime at a rate of 100 – 300 kg/t of liquid, complete hygienization was obtained during 2 days.

For sanitary and technological reasons, application of soda lye seems to be the best choice, but in the economic aspect, the effectiveness of lower NaOH rates should be tested.

Waste Management and Biogas Production

Ecological Studies on the Hazard Uses of Human Sewage at Egyptian Environment

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Summary: Human sewage wastewaters and sewage sludge has been utilized in areas around Assiut City, Egypt, for irrigation as a source of plant nutrients and soil amendments. The problem of this practice is the preaence of considerable concentrations of heavy metals which considered harmful to plants and animals.

Samples of water [5,10], Soil [4,7] and dietary green pastures [5,9] were collected from non-polluted and polluted areas respectively. Blood samples were collected from control [12] and exposed [25] buffaloe calves 6 – 9 months ages. Human sewage was the source of pollution at some villages near Assiut. Non polluted areas were clean and irrigated with water from River Nile. lead, cadmium, iron, copper and Zinc were determined using atomic absorption spectrophotometer.

Significant elevation ($P < 0.05$) was reported in mean values of lead 0.05 ± 0.001, 1.37 ± 0.03 and 14.12 ± 2.11 ppm in water, soil and dietary plants respectively at polluted areas. The same trend was observed in cadmium. Slight elevations were reparted in the values of iron, copper and zinc in dietary plants at polluted areas. Blood of exposed animals had higher concentration of lead (> 6 folds) and cadmium (> 9 folds) than corresponding control values. The biometals Fe, cu and znc had lower values in blood of exposed animals.

Key words: human sewage, buffalo calves, heavy metals

Introduction

Sewage wastewaters and sewage sludge has been utilized in areas around Assiut, Egypt, for irrigation as a source of plant nutrients and soil amendment. The problem associated with this practice is the presence of considerable concentrations of heavy metals which considered harmful to plants and/or animals [5]. Heavy metals has always occupied a central place in nature and represent an environmental hazard, because once they enter the environment, they cannot be destroyed [15]. Animals allowed to graze on or fed with contaminated pastures or fodders are exposed to various health hazards. [12]. The current study was planned to show the hazard effects of human sewage on the ecological constituents of the environment.

Material and methods

Samples of 10 ml water [5, 10], 20 mg soil [4, 7] and 0.5 kg of green forage [5, 9] were collected from non polluted and polluted areas respectively. Also, 10 ml blood was collected from each baffuloe calf (6 – 9 months age) healthy control group [12] and exposed group [25]. The exposed calves were living on polluted pastures irrigated with water containing effluents of sewage water. Lead, cadmium, iron, copper and zinc contents were determined in all collected samples [6, 14].

The obtained data were statistically analyzed [10].

Results and discussion

In the current work, water, soil and dietary planta at the polluted areas had significant higher concentration of Pb and Cd (> 11 folds, > 2 folds), (> 6 folds, > 8 folds) and (> 2 folds, > 5 folds) respectively as compared with values of non-polluted areas (Table 1). These findings were supported by [1,2]. The reported increase in Pb and Cd values in water, soil and dietary grasses were mainly due to environmental pollution with sewage wastewater, sewage sludge and other industrial effluents.

There are no obvious differences between biometals (Fe, Cu, Zn) values in water and soil samples at both areas (Table 1). On the other hand, marked elevations are reported in values of biometals in dietary plants. our data were similar to [5].

Significant elevation was reported in mean values of pb (> 6 folds) and Cd (> 10 folds) in blood plasma of exposed animals as compared with the control (Table 2). As for many elements levels in tissues are largely depent on metals content of forage [7]. In our work, blood Pb, concentration did not reach the toxic level but Cd slightly exceeded. This agree with [3]. It has been mentioned that Cd was efficiently absorbed than Pb by digestive system [13].

Table 1 Meatals concentration in water, soil and dietary animal grasses at the studied areas

Areas	Metals concentration (ppm)				
	Pb	Cd	Fe	Cu	Zn
			Water		
Polluted	0.05 ± 0.001**	0.03 ± 0.01*	0.10 ± 0.001	1.70 ± 0.03	3.71 ± 0.21
Non-polluted	0.004 ± 0.001	0.01 ± 0.001	0.01 ± 0.001	1.60 ± 0.01	2.02 ± 0.03
			Soil		
Polluted	1.37 ± 0.03**	0.09 ± 0.005*	10.19 ± 1.01	3.27 ± 0.05	5.14 ± 0.31
Non-polluted	0.22 ± 0.01	0.01 ± 0.001	9.16 ± 0.90	2.61 ± 0.04	4.62 ± 0.13
			Dietary plants		
Polluted	14.12 ± 2.11*	2.15 ± 0.11**	310.1 ± 34.01	27.16 ± 2.71	32.14 ± 2.81
Non-polluted	6.16 ± 1.31	0.42 ± 0.01	202.0 ± 52.1	18.12 ± 1.90	26.11 ± 2.02

Significant * $P < 0.05$, ** $P < 0.01$

Table 2 Average heavy metals concentvation in blood of buffaloe calves

Animals	Blood elements				
	Pb(ppm)	Cd(ppm)	Fe(μg/dl)	Cu(μg/dl)	Zn(μg/dl)
Control	0.023 ± 0.013	0.047 ± 0.029	130.4 ± 9.92	89.35 ± 6.35	100.2 ± 4.11
Exposed	0.151 ± 0.012*	0.48 ± 0.05*	97.71 ± 5.23*	69.78 ± 4.45*	89.2 ± 3.14*

Significant * $P < 0.05$

In spite of the satisfactory amounts of biometals in the diet, their levels were significantly lowered in blood plasma of buffaloe calves at polluted areas as compared to those at non-polluted (Table 2). It was suggested that an interaction between heavy metals and biometals may occur [9]. The decreased iron in blood of exposed animals could be attributed to the interference of Cd with iron absorption from intestine [8]. Also, Cd bind to liver ferretin that present in intestinal mucosa and involve in mucosal uptake and transfer of iron In a number of biological processes Cd interacts with Cu and Zn [4, 11].

Conclusion

The hazard use of sewage sludge as a fertilizer to provide growing plants with essential nutrients must be controlled because of the potential of unwanted constituents such as heavy metals. The significant elevation in blood heavy metals is a good index for hazardous accumulation of these metals in various body tissues.

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A Reduction of Extended-Spectrum Beta-Lactamase Carrying Bacteria (ESBL), Methicillin-Resistant *Staphylococcus aureus* (MRSA) and Vancomycin-Resistant *Enterococci* (VRE) by Biogas Plants?

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Summary: This paper describes the cultivation-dependent and molecular biological based detection of Extended-spectrum beta-lactamase carrying *Enterobacteriaceae* (ESBLs), of Methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant *Enterococci* (VRE) in manure from livestock husbandry used as input samples of biogas plants. In parallel, the respective output samples were investigated. The aim of the project is to determine the potential of biogas plants to eliminate ESBLs, MRSAs, and VREs to reduce the risk of their transfer to surface and ground waters.

Introduction

The widespread application of veterinary antibiotics in intensive animal husbandry lead to an increasing occurrence of antibiotic-resistant bacteria. These antibiotic-resistant "potentially" pathogenic bacteria can be released through manure into soil, ground and surface waters and can enter the human food chain via this pathway. In the era of "green energies", alternatively uses for manure can be found as input material of biogas plants to produce biofuels. In Germany more than 5900 biogas plants produce biofuel mainly via a co-fermentative process of energy crops mixed up with manure. The transmission of antibiotic-resistant pathogenic bacteria through biogas plants has not been investigated in detail so far. We aimed to determine the abundance of antibiotic resistant bacteria in manure samples used as input samples in biogas plants and investigate the potential of biogas plants which may have the function as a biotechnological barrier to prevent or reduce the transmission of antibiotic-resistant bacteria to soils, ground and surface waters. The project is focused on the isolation, characterization and molecular detection of extended-spectrum beta-lactamase carrying *Enterobacteriaceae* (ESBLs), Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Vancomycin*-resistant Enterococci (VREs). In the first phase of the project, input (cow-, pig-, chicken and horse manure) and output samples of 15 biogas plants in Germany were investigated.

Material and methods

A total of 15 biogas plants located in three different regions of Germany were investigated between March to October 2012. *Bacteria* were extracted from input and output samples of biogas plants using a sodium pyrophosphate-based extraction. Serial dilution of the extracted bacteria were plated in triplicates on ESBL, MRSA and VRE CHROMAgar (MAST Diagnostica GmbH) and incubated for 24 hours at 37℃. Colony forming units per g dry weight were counted and the morphologically most abundant colonies were characterized in more detail. Respective isolates were identified by 16S rRNA gene sequencing and screened for resistance genes using specific multiplex PCR systems. The screening for CTX-M, TEM, and SHV ESBL genes was performed by a Multiplex-PCR established by Monstein et al. (2010). All detected ESBL genes were sequence and used to determine the ESBL type. All ESBL carrying bacteria were additionally investigated with a specific antibiogramm panel containing 12 veterinary medicine relevant antibiotics including β-lactam antibiotics (+/− the ESBL inhibitor clavulanic acid), a fluoroquinolone, sulfonamides, a tetracycline and a macrolide. The screening for MRSA was performed using a Multiplex-PCR established by Poulsen et al. (2003) was used containing a primer system for a *S. aureus*-specific nuclease and the *mecA* gene responsible for methicillin resistance. The VRE screening was performed with a Multiplex-PCR including primer systems for five different *Vancomycin*-resistance genes, vanA, vanB, and vanC1 to C3 (Kariyama et al. 2008). The same multiplex-PCR primer systems were also used to detect ESBLs, MRSAs, and VRE resistance genes directly in input and output samples of biogas plants after DNA extraction. The efficiency of the cultivation-

dependent and molecular-based analysis was investigated by spiking of manure with defined numbers of ESBLs, MRSAs, and VREs cells (1×10^3, 1×10^5, and 1×10^7 cells).

Results and discussion

The direct cultivation on ESBL and VRE CHROMagar showed in general higher CFU per g (dry weight) in input samples compared to respective biogas plant output samples. On ESBL CHROMagar characteristic pink (presumptively ESBL carrying *E. coli*) and blue colonies (presumptively ESBL carrying *Klebsiella*, *Citrobacter*) were only determined in input samples but not in output samples of biogas plants. VRE characteristic pink colonies on VRE CHROMagar were also reduced in output samples but no CFU reduction occurred on MRSA media. A total of 56 abundant colonies from ESBL CHROMagar were phylogenetically identified and screened for ESBL genes. 46% of the isolates, which were derived from input samples, were affiliated to the *Enterobacteriaceae* (only 4% in the output samples). Other isolates cultured from ESBL CHROMagar were allocated to different genera of the *Pseudomonadaceae*, *Moraxellaceae*, *Alcaligenaceae*, and *Brucellaceae* differing in their abundances in input and output samples. A total of 56% of the *Enterobacteriaceae* from the input samples were identified as *E. coli* containing CTX-M-1, 2, or 9 type and/or TEM-15 ESBL genes. SHV ESBL genes were not detected at all. A *Morganella* sp. and an *Enterobacter* sp. were identified to carry CTX-M-1. In the output samples only one *Achromobacter* sp. containing a TEM-15 was detected. However ESBLs were directly detected by plating on ESBL CHROMagar plates a specific pre-enrichment increased the detection in input samples but again no ESBLs were detected in output samples. Antibiogramms showed that all ESBL carrying bacteria also showed an ESBL characteristic inhibition by clavulanic acid and a high resistance to several of the other investigated antibiotics. The direct detection of ESBL genes in DNA extracts of input and output samples from biogas plants gave only slightly positive results for TEM and partially for CTX-Ms (again no SHV was detected), but after a ESBL specific pre-enrichment CTX-M and TEM genes could also be detected in the pre-enriched input and output samples. A spiking with ESBLs carrying *E. coli* showed that a total number of 1×10^3 cell gave a slightly positively result compared to 1×10^5 cells which could clearly be detected.

A total of 22% (input) and 8% (output) of the VRE CHROMAgar isolates were identified as *Enterococci* but no vancomycin resistance genes could be detected. Other bacteria, which were also grown on VRE CHROMAgar were affiliated to the *Caulobacteraceae*, *Lactobacillaceae*, *Sphingobacteriaceae*, and *Acetobacteraceae*. Vancomycin-resistance genes were also not detectable in DNA extracts form input and output samples of biogas plants. Several colonies from the MRSA CHROMagar plates were screened for the presence of MRSA but without success. The detection of MRSAs in DNA extracts from input and output samples also failed. Spiking experiments clearly showed that 1×10^5 MRSA cells per g manure could not be detected on CHROMagar media because the MRSAs were overgrown by several other bacteria including *Bacillaceae*, *Planococcaceae*, and *Micrococcaceae*. A pre-enrichment procedure slightly modified to the method established by Johne et al. (2012) showed that MRSA could be detected after subcultivation of MRSA CHROMagar and molecular biologically in DNA samples extracted from input and output samples after the pre-enrichment. The molecular biological detection by Multiplex-PCR indicated that MRSAs are present in both input and output samples of biogas plants.

Conclusions

The results obtained by cultivation on ESBL and VRE CHROMagar gave a first indication that technological process in biogas plants seemed to reduction or even eliminate ESBL and VRE resistant *Enterobacteriaceae* and *Enterococci*. Especially for ESBLs a reduction of ESBL carrying bacteria was clearly indicated. Our data also showed the necessity to establish specific pre-enrichment procedure to enhance the detection efficiency of ESBLs, MRSAs, and VREs by cultivation-dependent but also for cultivation independent detection.

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Increasing the Effectiveness of a Holding Company for Organic Cow Milk Production by using New Biogas Substrate

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Summary: Up until now, studies on the production of biogas from manure have shown the prospects of this biotechnology as an opportunity for utilization of the energy in waste biomass as well as an opportunity to obtain a decontaminated product /compost/ with an optimal chemical composition/macro- and micronutrients content, quantity of water-soluble fractions/to improve soil fertility. This paper presents a model of a holding company for organic cow's milk production and energy from a renewable energy source – a mixture of manure and energy crops; for the first time the possibility of integrating two types of biotechnology (lactic-acid fermentation) and anaerobic decomposition of the substrate which includes silage from *Paulownia elongata* is being considered.
Key words: *Paulownia elongata*, silage, manure, organic cow milk, biogas

Introduction

In previous studies /Baykov et al. 2009/ the possibility of creating holding companies for the biological production of cow's milk combined with an installation for anaerobic decomposition of manure was proved. Using this biotechnology an integral environmental and economic impact is achieved since part of the energy contained in manure is converted to gas fuel energy /biogas/ from which electricity is produced; in addition a decontaminated product /compost/ with an optimum degree of mineralization of its organic matter and an optimal (for plants) balance between macro- and micronutrients is obtained. Up until now attempts included different mixed substrates of manure in order to balance the amount of biogenic macronutrients and meet the requirement to not exceed the annual dose of 170 kg of nitrogen per 1 ha of the holding in organic production. The installation for the production of biogas as a part of the technological chain for organic cow's milk production allows eliminating the risk of introducing pathogens into the soil; in addition the optimal amount and balance of biogenic nutrients for plants in manure is preserved since only part of the hydrogen, oxygen and carbon (which are not limiting for the agrocenosis) is eliminated by the obtained mixture of methane and carbon dioxide (biogas). An important advantage of our technologies for biogas production is the mineralization of the substrate by 48% – 64%, which allows the plants to be provided with nutrients in an accessible form /soluble fractions of nitrogen, phosphorus and other/, combined with slowly-degradable organic compounds, which are a source of the same elements. The use of energy crops as material for the production of biogas is rational in view of the amount of gas fuel produced in relation to its cost price. In the experiments up until now we have demonstrated the advantages of integrating two biotechnologies lactic-acid fermentation and anaerobic decomposition and use of corn as part of the substrate for biogas production. In this paper we present the possibilities of using *Paulownia* as an energy crop. So far it has been experimented as a forage crop or for the production of fuel pellets [1, 5, 6].

Material and methods

For the purposes of the experiment, silage of 2 energy crops is prepared: corn and *Paulownia*. The lactic-acid fermentation is carried out as the fresh plants are transported to the testing grounds where they are shredded to particles of 0.7 cm – 1 cm and silage is then prepared from them. Thus cut material is stuffed tightly into silage pits. For proper running of the lactic-acid fermentation the following conditions are met: absence of oxygen [anaerobic conditions] and appropriate pH and temperature. For this purpose after a good reconsolidation the silage pit is covered with a foil sheet of 0.4 mm thickness and is then covered with dirt.

For the purposes of the experiment cattle manure from livestock reared without bedding and litter from broiler production was used.

Mixtures with different proportions of manure (30% – 50%) and maize or *Paulownia* silage (50% – 70%) are prepared; they are ground to particles of 2 mm and are diluted with water to 7% content of the dry matter. Experimental laboratory installation developed by the team is

used; it allows simultaneous experimentation with several substrates (Fig. 1). Based on the mathematical modelling method of Chen & Haschimoto[3] the optimal technological parameters are determined: dry matter content in the substrate of 7%, mesophilic temperature regime (33℃), time in the fermenter-15 days. The amount of obtained biogas is recorded daily by the graduated scale of the receiver while its qualities-by Draeger. The chemical composition of the substrate is determined by routine methods detailed by us in previously published publications [2, 4].

Results and discussion

The obtained results for the chemical composition of the four substrates used in the experiments are presented in Table 1. Significant differences in the amount of dry matter are found: it is highest in maize silage (49.43%) and the litter from the broiler production (43%). In this series of experiments the dry matter content in the *Paulownia* silage is 22% but it is possible, and results were achieved in the production of silage with 54% dry matter content. When optimizing the technologies for biogas production, a key parameter is the quantity of the organic matter: its content is highest in the *Paulownia* silage (93% of the dry matter), in the maize silage (88%), in the litter from broiler production (85% of the dry matter) and in the cattle manure (77.9%).

Table 1 Chemical composition of different materials for biogas production

Parameters /in%	Cattle manure	Litter from broiler production	Corn silage	*Paulownia* silage
Dry matter	11.78 ± 0.12	43.00 ± 0.15	49.43 ± 0.42	22.00 ± 0.17
Organic matter	9.18 ± 0.11	36.55 ± 0.62	43.49 ± 1.02	20.46 ± 0.44
Organic carbon	34.60 ± 0.52	32.49 ± 0.48	34.60 ± 0.88	42.94 ± 0.64
Nitrogen	3.44 ± 0.17	6.82 ± 0.22	1.09 ± 0.16	1.96 ± 0.22
C : N	10.0	4.8	31.7	21.9

It is estimated that for all four substrates the organic matter is within the optimum. Another indicator of which biogas production depends is the ratio C: N. The different authors' data for the optimal values of an index are very different: from 4 – 10.1/Braun, 1981/, to 45 with an optimum of 20 – 30 /Hawkes, 1984/[3]. When this key indicator of the effectiveness of biogas production is considered, all four substrates meet the requirements. Our previous experiments have shown that the high level of nitrogen in the litter is a reason for inhibition of methanogenesis at an independent use of this substrate. For this reason we use mixed substrates of manure in which the litter is a source of nitrogen and the cattle manure of carbon. When using energy crops silage, the energy capacity of the substrate (measured by the ratio C : N) is increased several times which is associated with significantly greater biogas production.

The data presented in Table 1 should be assessed in other ways too. We have developed modules of holding companies for biological production of cow's milk which include installations for biogas production. The modules are from 10 to 150 dairy cows in need of pastoral areas of 1 ha for 2 cows. Experiments show that enough biogas is produced from manure from one cow to provide 1 kWh of power to an electric generator. Under current legal regulations it is appropriate to build installations of up to 30 kWh which requires 150 dairy cows. According to Regulation №35/2001 on organic production in livestock breeding, it is necessary to ensure 2 ha of pastures for 10 cows. The purpose of this regulation is to limit the pollution of the soil when the quantity of the manure doesn't exceed 170 kg/ha per year. For the agrocenosis' management we determine the quantity of the introduced compost by shaping the whole technological chain: establishing plantations of maize (mainly for fodder) and Paulownia (50% of the substrate for biogas production). Based on the information on the nitrogen content in both crops (plants) we calculate the necessary amount of nitrogen to be introduced into the soil in order to achieve maximum productivity (nitrogen is a limiting element in agrocenosis) without risking contamination of soil and groundwater with nitrogen compounds.

In our study the plant material is used after ensilage. The combination of two biotechnologies: lactic-acid fermentation of energy crops and anaerobic decomposition of mixed substrate, increases the efficiency of biogas production as in ensilage a part of the difficult degradable organic compounds is degraded.

In the production of energy crops in the holding company a basic requirement is respected: a predetermined area for the holding company spatially isolated from the other agrocenosis. When using one energy crop, such as maize, there are risks for the soil typical of monoculture farming: reduction of soil fertility and soil degradation.

Our model uses two plants which are exploited in rotation: the compulsory area under Regulation №35 is divided in 2 sections, one of which is planted with the perennial energy crop *Paulownia elongata*. The areas with this introduced species are exploited for an average

of 4 years; each year three batters are received, they include leaves and stems from which silage is made. The remaining area is planted with maize from which silage for the cows is prepared and in case there is excess-for the production of biogas. According to literary data [1] *Paulownia elongata* improves soil structure and it is rational to cultivate it for four years after which rotation of the crops is made.

Conclusions

In agro-environmental systems' management a main requirement is limiting the linearity of the technologies. In the farms for organic cow's milk production two products are obtained: cow's milk and manure. Our research shows that the introduced to the system energy is distributed equally among them. For that reason the organic fertilizers are considered and used as an energy source. Different technologies for obtaining energy from manure via burning are developed around the world. On the other hand manure contains all the necessary biogenic nutrients for the plants, but is a risk factor for agrocenosis because of the presence of pathogenic microorganisms. The only possibility for effective use of the energy and storage of the biogenic nutrients is the anaerobic decomposition of manure. To increase the efficiency of biogas production we propose a model of a holding company for biological production of cow's milk in which two energy crops are grown: maize for the production of fodder and Paulownia for biogas production and to improve soil's structure. We also offer modules of holding companies with a capacity of 10 to 150 cows in which the cost-effective (economically justified) limit of 150 cows for reaching enough power for the power generator to produce 30 kWh of energy is not exceeded. The required area of 75 hectares is divided in two sections of 75 ha; in one the perennial crop Paulownia is planted while in the other-maize. Crops are rotated after a period of 4 years.

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New Materials for Biogas Production in Livestock Farms in Bulgaria

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Summary: A comparative study was conducted for the production of biogas utilizing raw materials that are new for Bulgaria. The study included three types of beetroot: sugar beet, red beet and fodder beet in two variants. First variant: heads + leaves, Second variant: heads. For all the raw materials the following content was determined: dry matter, organic matter, nitrogen and crude ash (minerals content, including some toxic elements). The results of the analysis show that the highest values of dry matter were found in sugar beet in both variants: 20.61% and 28.12% respectively. In terms of organic matter content, the tendencies of higher values in sugar beet remain (13.51% and 26.21% respectively for both variants). Nitrogen content is highest in fodder beet – 2.48% and 1.90% respectively for both variants, and it is the lowest in sugar beet – 1.65% and 1.30% respectively for both variants. A tendency for higher crude ash content in fodder beet is observed; the content of toxic elements such as cadmium and lead are highest in both variants. Sugar beet stands out with best values for each indicator characterizing the production of biogas.
Key words: beet – species and parameters characterizing the production of biogas.

Introduction

Biogas markets around the world have expanded significantly in recent years with many countries seeing biogas production and development becoming extremely important. The European biogas sector consists of thousands of installations and countries such as Germany, Austria, Denmark and Sweden are among the technical leaders.

There are three main sources for biogas production: landfills (production of 35.9%), urban and industrial wastewater treatment plants (12.1%) and specially built installations which use energy crops as materials (52%).

Most biogas systems in Asia use simple technology and therefore are easy to manufacture. In China, for example, it was estimated that 18 million household biogas reactors produce 145 billion m^3 of biogas. On the other hand in the U. S., Canada and many Latin American countries a modern biogas production sector is developing; the developments aim to improve the operational process by using new combinations of feedstock.

In this regard, studies of new energy crops for the production of biogas are being conducted in many countries (especially in Europe); this is done in order to improve the productivity of the energy crops and assess their biogas potential.

Along with manure from livestock rearing, the decentralized CHP plants have great potential; the treatment of wastewater and solid waste as well.

In the next 10 – 20 years an increase in the use of energy crops is expected; 20% – 30% of arable land is expected to be used for biogas production. Maize (corn), sugar beet and other energy crops will play an important role in the production of bioenergy [4,5].

In previous studies [6,7] we made a characteristic description of some energy crops in terms of key indicators characterizing the production of biogas.

With the present study we aimed to characterize the new energy crop beet of three different species, used in two variants: tubers + leaves and tubers only in basic indicators of importance in biogas production.

Material and methods

With regard to the established objective, samples of beet from different parts of the country were taken. The samples included: 1. Sugar beet in two variants: tubers + foliage and tubers only; 2. Red beet: tubers + foliage and tubers only; 3. Fodder beet: tubers + foliage and tubers only. The collection and formation of the samples were conducted according to EU Regulation 152, while the preparation for analysis of the samples under ISO 6498. All samples were analysed for moisture content [ISO 6496] in order to determine the dry matter content. Determination of the crude ash content was carried out using the dry ashing method [ISO 5984], whilst individual elements were determined by atomic absorption spectrophotometry (AAS) with different wavelengths [AOAC, 2007]. The amount of nitrogen was determined by the Kjeldahl method [ISO 5983].

The values obtained for the individual indicators

were processed statistically.

Results and discussion

The indicators that characterize beet as a source of biogas are presented in Table 1. Significant differences in dry matter content in the different beet species are observed. With the highest content of the three species is sugar beet in both variants: tubers + foliage and tubers only – 20.61% and 28.12% dry matter respectively. Regarding the dry matter indicator, fodder beet has the lowest values in both its variants (13.15% for tubers + foliage and 15.98% for tubers only). Sucrose is 94% of beet's dry matter. Species with high sucrose yield are important for the production of biogas. Regarding the organic matter indicator, sugar beet retains its high percentage in both analysed variants: 13.51% for tubers + foliage and 26.21% for tubers only. Fodder beet has the lowest values of organic matter in both variants (4.41% in the first variant and 9.78% in the second). In terms of the dry matter and organic matter indicators, garden beet (red beet) occupies a mid-position in both analysed variants. In a previous study comparing tubers of sugar beet and red beet, higher content of organic matter (15.9%) was determined in sugar beet, which allows its addition as raw material to the main substrate silage corn. The period of full fermentation of sugar beet is relatively short: in the range of 15 to 20 days (compared with silage corn about 90 days). In addition methane yields per hectare are at about 20% more than that of silage corn (54 t/ha corn correspond to 80 t/ha sugar beet) [7].

Table 1 Composition of energy crops

Indicators	Dry matter(%)	Organic matter(%)	Nitrogen(%)	Ash(mg/g)
Sugar beet tubers + leaf mass	20.61 ± 1.03	13.51 ± 0.68	1.65 ± 0.21	71 ± 1.98
Sugar beet (tubers)	28.12 ± 0.29	26.21 ± 1.13	1.30 ± 0.07	19 ± 0.18
Red beet tubers + leaf mass	17.90 ± 0.91	10.50 ± 0.48	2.37 ± 0.15	74 ± 2.39
Red beet (tubers)	18.21 ± 1.12	15.21 ± 0.96	2.25 ± 0.09	30 ± 1.67
Fodder beet tubers + leaf mass	13.41 ± 1.15	4.41 ± 0.11	2.48 ± 0.11	90 ± 2.14
Fodder beet (tubers)	15.98 ± 0.78	9.78 ± 1.01	1.90 ± 0.31	52 ± 1.49

The data for nitrogen content show that sugar beet has the lowest values in both variants: tubers + foliage 1.65% and tubers only – 1.30%. The differences in nitrogen content in the two variants of red beet (tubers + foliage and tubers only) are small; they are more significant in fodder beet in both analysed variants. Testing in Germany helps to support our results having studied different species of sugar beet. It has been determined that for the production of biogas two species of sugar beet are used and those are: LUKAS Z-type and BENNO N-type as their main advantage is the low levels of nitrogen [8,9].

The ash content in the analysed species of beet varies from 19 mg/g to 90 mg/g. It is noteworthy that high levels of ash are observed in the first variant (tubers + foliage) in all three species of beet. Sugar beet's tuber has the lowest ash content – 19 mg/g which is 15% and 27% less than that of red beet and fodder beet respectively.

Table 2 Content of elements in different types of beets

Elements(mg/kg)	Fe	Pb	Cu	Mn	Zn	Cd
Sugar beet (tubers + leaf mass)	926	2.5	8.5	72.5	18.3	0.80
Sugar beet (tubers)	638.0	3.0	15.8	75.0	70.5	0.70
Red beet (tubers + leaf mass)	880.0	3.5	10.5	130.0	19.3	0.70
Red beet (tubers)	187.0	1.6	1.1	70.0	25.5	0.65
Fodder beet (tubers + leaf mass)	181.0	3.7	0.5	20.0	26.5	1.40
Fodder beet (tubers)	195.5	3.3	8.0	59.0	15.5	1.10

Table 2 presents the results for the presence of certain elements in the three studied species of beet. Of the analysed toxic elements – lead and cadmium, it can be seen that their values are highest in fodder beet in both samples (tubers + foliage and tubers only). In respect of the other analysed elements a large divergence is observed in both variants of all three species of beet. In this case the studied toxic elements' (cadmium and lead) values are below the permissible critical values for sugar beet and red beet.

Conclusions

Based on the conducted analysis of these three species of beet, each of which represented in two variants: 1st – tubers + foliage and 2nd – tubers only, we can make the following conclusion:

- The main indicators characterising the production of biogas – dry and organic matter are of the

highest values in sugar beet in both variants. This combined with the high yields of fresh biomass per unit of area, rhythm is ensured in the biogas production.

- In comparison with the other two species of beet: red beet and fodder beet, sugar beet has the lowest content of nitrogen both in the tubers and in the whole plant – tuber + foliage.
- Sugar beet has the lowest ash content in both variants. A similar tendency is observed in the analysed toxic elements – cadmium and lead.

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Risk Assessment of Nitrogen and Phosphorus Transfer after Manure Land Application: A Review of the Main Influencing Parameters

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Summary: The two major pathways of N and P loss from a plot of land receiving manure to water resources are leaching and runoff. The risks of contamination of surface water by runoff are associated to the sensitivity of the plot to runoff and N, P availability/mobility during runoff which depends on the rate of manure applied, climatic conditions, application technique and soil conditions (humidity,...). N, P loss by runoff following manure spreading could be strong in winter, spring and summer during heavy rainfall. Therefore the risk of transfer by runoff should be defined according to the cover of the plot rather than the application period. However, the likelihood that runoff water reaches rivers is not clearly established as it also depends on various parameters (plot characteristics, distance to river, tillage,...). The water contamination due to infiltration is also complex with the same rage of factors influencing runoff loss. In addition, leaching loss is also related to nutrient cycling and their transport through the soil profile.

Introduction

In most countries of Europe livestock manure is managed mainly by spreading to agricultural plots. The effects of such management practices on the environment, most of them related to emissions of nitrogen (N) and phosphorus (P) to water resources, have led to national or european regulations concerning the N, P level of spread manure, periode or distances from waters. However, the value used in these regulations (170 kg N/ha in the case of the European Nitrates Directive) are often discussed or disapproved because there are not always based on scientific evidence. N, P loss from of an agricultural plot are linked to the complex and natural processes of the water cycle which have been studied in detail and documented elsewhere. The main processes responsible for this loss are runoff and leaching.

Nitrogen and phosphorus loss after manure spreading

N loss by runoff concerns mainly ammonium (NH_4^+) and can be significant depending on the N applied level and the time between application and rainfall events [1, 2]. N runoff is estimated at 2% -4% of Napplied but greater loss can be expected with extreme rainfall (storm) and slope of 15/20°[3]. NO_3^- runoff was also measured [4] but was extremely low. N leaching is by NO_3^- and are generally between 1% and 25% of N applied depending on the type of effluent (liquid > solid) and the application technique[5, 6]. Soil and culture type also influence the level of loss. Loss of 50% of N applied nitrogen were observed on a sandy grassland with cattle manure [7]. NH_4^+ leaching is low due to the conversion in nitrate and the retention by soil particles. P loss by runoff can reach 10% -20% of applied when an event occurs shortly after spreading [8, 9] with loss decreasing during subsequent rainfall events [10]. Few data are available on the P loss by leaching after manure spreading.

Factors influencing the nitrogen and phosphorus loss

N, P loss is influenced by several factors including manure characteristics, soil type, time and manure rate, spreading practices and land use and management practices.

Manure: Physical and biochemical characteristics influence N and P loss following manure spreading. N loss is related to the mineralization and organization processes of N soil which depend on the nature, degree of stability and nitrogen effluent spread [11]. NO_3^- leaching is directly proportional to the amount of readily available nitrogen (NH_4^+, uric acid) with NO_3^- loss greater with poultry manure for example [7]. Level and particulate fraction of dry matter may also influence N runoff by impacting the manure rheological properties and infiltration [12]. High loss in NO_3^- could occur with the spreading of high manure rate but without linear relationship between N loss and N applied [13] due to the alteration of N and residual soil NO_3^- which is linked to previous crop and repeated manure spreading. The manure rate applied also influences phosphorus loss if rainfall events occur soon after application [14] but the P capacity sorption and the runoff aptitude of the soil also

interfere.

Period of application: The period of application affects the loss due to crop or because of climatic conditions (temperature, rainfall,...). Manure spreading under European conditions in April/May can lead to significant loss of nutrient to surface water or groundwater with soil susceptible to erosion during some rainy spring [13,15]. Application in June/July limits nutrient loss due to plants growing and less soil infiltration. Spreading in August/September leads to a high risk if no cover crop is established. Runoff loss depends of the intensity or cumulative rainfall as well. These parameters are independent of the period of application. Runoff loss are much greater when rain occurs soon after application and the effluent is left on the surface [16,22]. The influence of spreading period on P loss is less studied than N loss. In Canada it is recommended to spread manure only in June/July while no P loss increase in autumn was observed when manure is incorporated by ploughing immediately after [17].

Type of soil: Structure, texture and topography of the land influence N, P as it determine the runoff/infiltration aptitude of rainwater [13, 15]. Soil characteristics also interfere on N, P mobility by adsorption/filtration process. With same rainfall and agricultural conditions NO_3^- loss are greater with sandy or loamy soils compared to clayey soils. Wet soil increases the risk of nitrogen loss through leaching and runoff. P loss by runoff are high on a loam beating soil (susceptible to erosion) and steep slopes [18] while leaching loss are promoted on a sandy or drained clay soil without major influence of the slope [19].

Land plot management: Few scientific studies refer to the influence of the distance between the plot and the river on N, P transfer to water [20 – 22]. However, it is recognized that the risk is higher if the distance is short and the plot is bared or without field edges. Also, the distance impact on N, P transfer to streams depends on the local topography. Runoff seems to be amplified by the slope but studies establishing this relationship were generally carried out at laboratory scales [23,24]. N, P transfer are reduced by buffer strips especially in particulate form and the reduction depends on many factors (width, density and type of vegetation,...) [22].

Spreading technique: Some studies showed a N loss reduction when using an injection technique while others showed an increase or no effect compared to surface spreading [5, 25]. These contradictory results are linked to the fact that NO_3^- loss is influenced by many parameters (manure, tillage before spreading) that could modify the N loss pathways. P loss by runoff are limited by manure injection [17,26].

Tillage: Crops and farming practices greatly influence the N, P loss [27,28]. Crop residues interfere by altering the C and N cycle and by reducing erosion and runoff. Plowing across the slope also has a positive effect in reducing runoff, however this effect is variable depending on the land configuration.

Conclusions

This review summarizes the main parameters influencing the N, P loss by runoff and leaching after manure spreading. Some gaps and uncertainties exist on the combined effect of the various factors influencing the risk of contamination of water resources. Indeed, scientific studies generally focus on one or two parameters. it appears that the accurate assessment of risk spreading manure is relatively complex because it requires cross above factors such as agronomic factors (soil type, crop, spreading periods,...) and technical factors (uniformity of spraying, fertilizing conservation value).

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Compost and Fermentation Residues Used as Litter Materials in Dairy Farming

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Summary: In dairy farming, litter materials have an essential influence on health and production performance of animals and therefore, on the quality of the comestible milk. As straw has recently become an essential cost factor in dairy farming, alternative options are in demand. In this study, selected materials were tested for their hygienic-microbiological quality, i. e. compost barns, litter materials from dry fermentation residues as well as composted fermentation residues, composted garden and forest waste.

Representative collected composite samples from the litter materials are taken from commercial farms. From compost barns 20 collected composite samples (10 from surface and 10 in a depth of 40 cm) were taken. From fermentation residues five collected composite samples of the fresh material and five of the dry material were analyzed. These materials were not used as bedding material at the time-point of sampling. From composted fermentation residues five representative samples were taken from the cubicals equally from composted garden and forest waste. In addition, three collected composite samples were tested from this fresh compost material. All samples were quantitatively tested for coliform bacteria, enterococci, salmonellae, streptococci and staphylococci.

Investigated materials showed varying results. The average bacterial numbers for *E. coli* and coliform bacteria in compost ranged between 10^5 and 10^6 CFU/g, respectively. In fermentation residues the numbers were considerable lower. In fresh material coliform bacteria were below the limit of detection and in dry material below 1.44 CFU/g. In used bedding material enterococci were verified in average between 10^3 and 10^5 CFU/g. In comparison, 10^1 CFU per gram of enterococci were found in fresh fermentation residues and 10^4 CFU per gram in dry fermentation residues. Salmonellae were not found in any samples. There were only a sporadically appearance of streptococci and staphylococci.

In summary, the content of *E. coli* and enterococci was quite high in all compost material. Therefore, this material can be recommended in a limited way as litter material. The variation of the results might be due to the sampling scheme since contamination with animal's excrements could not be avoided.

Introduction

In dairy farming the bedding material is very important. However, economy, working aspects, animal welfare, and hygiene must be optimized in each farm. There are a lot of possibilities, e. g. traditional straw, mixtures of different other organic materials, but also practices without any bedding materials. One common aim of all these methods is to provide a long time of recumbency for the cows. This is very important for a high milk production. Moreover, it should be not too expensive and the workload should not be too high. Last but not least a high hygienic quality is critical for healthy animals and subsequently for secure and faultless comestible milk.

Price of straw is increasing constantly. In addition, there are also a lot of problems in systems without bedding materials in cattle husbandries. Therefore, some alternatives are searched.

In this project selected alternative materials were tested particularly with regard to their microbiological contamination and hygiene. The first husbandry system uses a continuing composting process, so-called compost barns. These barns should combine consumer requirement for animal welfare with high demand for milk quality and limited workload. Another innovative possibility is the usage of dried fermentation residues from digester. Through the increasing number of digester, there arise high amounts of fermentation residues. Possibilities for the usage of these materials are searched. One possibility is to use the dried material as bedding material in cow barns.

Furthermore, there is a look at other compost materials, i. e. composted garden and forest waste as well as composted fermentation residues of communal biological waste recycling. The possibility of these materials as bedding material will be examined.

Material and methods

This project looked at two compost barns. One of these uses wood chips and the other wood shavings. Fermentation residues were derived from one biogas plant

in Baden-Württemberg. A producer composts garden and forest waste and scatters the prepared compost which is mixed with feed lime as litter material. Another farmer purchase composted fermentation residues of communal biological waste recycling.

Sampling was performed on a total of five commercial farms. The samples from compost barns were taken at two farms in Austria, i. e. 20 collected composite samples. In each case these are mixtures of five primary samples. Ten are taken from surface and ten in a depth of 40 cm. As sampling location there were selected representative areas all over the bedding area.

One of these farms needs the barn in winter month, about October till May, and needs as base wood chips. The other farm needs the barn all over the year and uses wood shavings.

Fermentation residues were derived from one biogas plant in Baden-Württemberg. Five representative samples consisting of five single samples were collected from fresh fermentation residues and from dried fermentation residues, respectively. These materials were not used as litter material, so far.

Composted fermentation residues from a loose-housing stable with cubicals in Switzerland were collected (five gross samples) and tested.

Ready compost derived from garden and forest wastes were mixed with feed chalk and used as litter material in cubicals in Switzerland. In this farm, samples were taken accordingly from the cubicals as well as from the fresh compost outside the stable.

All samples were quantitatively tested for coliform bacteria, enterococci, salmonellae, streptococci and staphylococci using standard culture methods.

Results and discussion

Compost barns

In compost barn with wood shavings the average contamination with *E. coli* was 4.44×10^6 CFU/g within the surface samples and 2.0×10^6 CFU/g within the samples taken in 40 cm depth, respectively. Therefore, it is slightly higher than in the compost barn with wood chips where average contamination was 1.09×10^6 CFU/g on the surface and 5.14×10^5 CFU/g in 40 cm depth. The bacterial counts for enterococci were lower. Average numbers ranged between 8.59×10^3 CFU/g and 3.25×10^5 CFU/g. Thereby, accounts in the barn with wood chips were also lower in comparison to the barn with wood shavings. Salmonellae could not be detected in any of the samples.

In the barn with wood chips staphylococci were detected in two samples on the surface and one sample in 40 cm depth. Streptococci were unverifiable.

In the barn with wood shavings staphylococci were detected in two samples on the surface only. Streptococci were unverifiable, too.

Fermentation residues

In fresh material *E. coli* were unverifiable and enterococci were verified to a amount of 2.4×10^2 CFU/g. After drying it increased. Salmonellae were not detected. In fresh fermentation residues, neither staphylococci nor streptococci were detected. In two samples of the dried material staphylococci, but no streptococci could be proved.

Compost

The average contamination with *E. coli* was 6.54×10^5 CFU/g and with enterococci 1.98×10^4 CFU/g.

In composted, but unused material *E. coli* was detected amounting between 9.2×10^0 CFU/g and 4.3×10^1 CFU/g. In used material the average content was 2.83×10^5 CFU/g. In unused compost the average content of enterococci was 2.24×10 CFU/g and in used compost 8.85×10^5 CFU/g. Salmonellae could not be detected verify in any of the samples. In one sample of composted fermentation residues streptococci could be verified. Staphylococci were detected in one sample of the composted garden and forest waste. Neither streptococci nor staphylococci could be detected in any of the other compost samples.

The contamination with enterococci and *E. coli* in the tested compost barns is relatively high, because the composting process is insufficient. The machining depth is too low, particularly in the barn with wood shavings; thereby the oxygenation is too low. Thus the activity of the microorganisms, which are important for the composting process, is blocked. It's positive that there were not detected any salmonellae and streptococci in this two barns. Staphylococci were detected just sporadically and these are also expected to be found in used litter material. In the fresh fermentation residues *E. coli* are below limit of detection and the content of enterococci were very low. Salmonellae, staphylococci and streptococci were also unverifiable. This can be traced back to the heating during the fermentation process. The contamination of the dried material was slightly higher, although there was a second heating during the dehydration. This indicates a contamination during transport or storage. It's possible that the tractor bucket was contaminated after carrying manure. When the fermentation residues were carried with the same tractor bucket, the material will be contaminated, too. Another possibility is a contamination by manure near the storage area.

The microbial contamination of the composted and interspersed fermentation residues lies in a similar range as the compost barns. But the composting process occurs in composting firm, hence it is assumed that the

composting process is preceded accurately and thereby the disinfection is achieved. So the high contents are caused by a later contamination during the storage near the barn and in the cubical.

The material, which the farmer composted by himself has low contents of *E. coli* and enterococci when it is still outside the barn. Besides, staphylococci, streptococci and salmonellae were unverifiable. From this, it is assumed that there was a good composting process. The used compost is comparable to the other samples, which were taken from the used bedding materials in cow barns. Thus the higher contents can only be explained by contamination with excrement of the animals. Also these contents are in an area, which is acceptable for dairy husbandry.

Conclusions

Generally, all analyzed materials are acceptable for dairy husbandry. Certainly all materials which were inside the barn are near the limit to be risky for udder health. It was unable to test dried fermentation residues, which were used as bedding material. Here akin results are due.

From the view of a safe condition for reasons associated with hygienic control of epidemics the compost materials must be complete sanitized and in particular the fermentation residues, which were used as litter material, have to come from a thermophilic actuated biogas plant or from such digester in which the substrates are pasteurized before the anaerobic fermentation. Only in this way it can be prevent that pathogens spread out unregulated. Particularly, this applies to the selling from fermentation residues as bedding material, because thereby separate farms will connect.

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Composting of Pig Faeces with Corn Stalks in China—Microbiological Examinations; Hygienic Aspects and Sanitation Capacity

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Summary: Composting is a possibility to produce a fertilizer in consideration of hygienic safety which could be transported to agricultural areas in regions far away from areas with a high pig production and density. The challenge was to determine the sanitation capacity of different variants (pig faeces and corn stalks, turning interval and cover) of composting processes, especially under winter conditions in Beijing, and to estimate the microbiological risk for human health, animal health and the environment. As test organism *Salmonella* Senftenberg W775 (H_2S negative) was used for microbiological examinations in the experiment. Inoculated test carriers were inserted in the rotting boxes in three different positions. In regard to the hygienic safety the temperature and other influences of composting procedure at each position in every box seem to be sufficient to inactivate the test organism in all six variants of composting.

Introduction

The use of organic matter (wastes) for sustainable reuse of nutrients is a very important issue all over the World, especially in countries with distinct agriculture and livestock production. China developed very fast over the last years with an increasing luxury and the raising demand for meat head to an increased pig production in China, especially in the Northern East of China around mega cities like Beijing. Increasing pig production in large scale pig farms head to enormous amounts of organic wastes (e. g. pig faeces, manure, slurry), over fertilization of agricultural areas and environmental pollution. In 2008 pig production worldwide achieve around 100 million tonnes of slaughtered pigs and China is leading with 48. 5 million tonnes, followed by European Union with 22 million tonnes and the U. S. A. with 10. 45 million tonnes ("Das meisteSchweinwird in China produziert", Agrarheute. com 2009). Intensified pig production around mega cities head to a decoupling from regions with intensive livestock production and areas of agricultural landscapes. Recycling of organic wastes in agriculture after appropriate biological treatment can produce valuable organic matter and be of great interest in countries where soils are depleted (HASSEN 2001). A variety of heavy metals and pathogens can be found in raw bio-wastes (STRAUCH 1996). Animal manure is a well-documented source of zoonotic pathogens, such as *Escherichia coli* O157:H7, *Salmonella* spp., and *Campylobacter* spp. (ERICKSON 2009). Pig manure can contain pathogenic micro-organisms creating the risk of spreading animal diseases and zoonotic agents. Pathogens can be spread from farm into and through the environment into the food chain via plants, food of animal origin, and into drinking water and may cause human infections (BOEHM 2009). Handling and use of organic wastes of animal origin are connected with risks for animal health, public (human) health and risks for the environment. New transmission routes of pathogens between rural and urban areas can be created through the use of organic waste products, it might be occur through aerosols, run-off from arable land to adjacent watercourse, contamination of groundwater, contamination of food and feed as well as because of vector animals such as birds and rodents (ELVING 2009). Sanitation of organic waste before application to soil is recommended because the pathogens may survive for extended periods in the soil environment (NICHOLSON 2005). Safety use of organic wastes as fertilizers demands a biotechnological treatment which guarantees a sufficient sanitation capacity. The sanitation capacity of composting process and the hygienic safety of the end product should be monitored and validated using measurement of temperature, insertion of test carriers with specific test organism (e. g. heat resistant *Salmonella* Senftenberg W 775) and following microbial examination of test carriers and end products, e. g. in accordance to the German Bio-Waste Ordinance. Aerobic, thermophilic composting is a common method to treat organic wastes. Composting is a possibility to produce a fertilizer in consideration of hygienic safety which could be transported to agricultural areas in regions far away from these mega cities. The challenge was to determine the sanitation capacity of different variants of composting processes, especially in cold season in Beijing, and to estimate the microbiological risk for hu-

man health, animal health and the environment.

Material and methods

Pig manure and corn stalks were used for composting in a ratio 1 : 7 (wet weight) and carried out in small rotting Boxes (1 m^3) as an half technical experiment in a farm building under roof. The six variants of composting differed in turning interval (no turning (Box 1 and 2), turning once every two weeks (Box 2 and 3) and turning once (Box 5 and 6) a week) and cover. Two boxes were the same in turning interval, one covered and one uncovered. The composting experiment ran over ten weeks, from end of November 2009 until beginning of February 2010, without forced aeration. The composition of our experiment and the bacteriological investigations were designed in accordance to the German Bio-Waste Ordinance (2011). As test organism *Salmonella* Senftenberg W775 (H_2S negative) (called "W775") and as indicator organism faecal streptococci were used for microbial examinations in the experiment. "W775" is a thermally stable and not pathogen *Salmonella* strain for human health (GARIBALDI 1969). Mixed input materials were inoculated with a suspension of test organism "W775" and put in sterile sacks. These served as test carriers and were prepared immediately before starting the composting experiment by inoculation with test organism. The test carriers were inserted in the rotting boxes in three different positions, representative for parts of the rotting boxes with different risks for hygienic safety. The firstlocation was the base, the centre and the margin of the box. In the trial the sampling plan included a sampling after 14, 49 and 70 days. During turning of composting boxes the test samples were taken out and inserted again after turning on the same position in the composting boxes. This should guarantee that the conditions are representative for the basis, the centre and the margin over the whole time of experiment. During the experiment qualitative and quantitative examinations were carried out to determine the presence of "W775" in control and test samples on day 0, 14, 49 and 70. Data loggers were used for temperature measurements every hour on each position of test samples during the whole time of composting experiment. The temperature, dry matter content and exhaust gas composition were regularly monitored. For qualitative detection of Salmonella there was a pre-enrichment of 50 g of each sample in 450 ml buffered peptone water. For quantitative detection 20 g of each sample were weight in into 180 ml of sterile sodium chloride solution (0.9%), shaked and ten folded diluted. For each dilution three parallel tubes with buffered peptone water were inoculated and incubated for 24 h (48 h) at 37℃. From pre-enrichment 0.1 ml was inoculated in selective enrichment broth Rappaport-Vassiliadis (RV-broth) and incubated for 24 h at 42℃. After incubation the RV-broth was streaked out onto XLD- (Xylose-Lysin-Desoxycholat) and BPLS- (Brilliantgreen-Phenolred-Lactose-Saccharose)-agar and incubated for 24 h at 37℃. Suspicious colonies were streaked out onto standard-I-agar, incubated for 24 h at 37℃, to receive pure colonies. These colonies were confirmed as "W775" by object slide agglutination with Salmonella specific test-sera. The number of "W775" was quantified using the most probable number-method.

Results and discussion

The numbers of "W775" in the control and starting sample in the beginning of experiment were $(1.5 - 4.3) \times 10^7$ CFU/g. The results show that "W775" was not detectable after 14, 49 and 70 days of composting in all of the tested test samples. In compare, "W775" was still detectable more or less without any decrease by orders of magnitude in the positive control samples over the whole composting period. In regard to the hygienic safety the temperature and other influences of composting procedure seem to be sufficient to inactivate "W775" at each position in every box in our test samples in all six variants of composting. Temperature is the most important factor to eradicate "W775" and when temperatures in the composting heap are 60℃, test organism "W775" will be eliminated within 10 h of composting (CEUSTERMANS 2006). Temperatures of 55℃ and higher were achieved during first week of composting and last for at least one up to three weeks. They correspond to the recommendations in national or international standards (e. g. German Bio-waste Ordinance). These results should be transferred into full technical composting plant. The results show that composting of pig faeces with corn stalks is a possibility to inactivate pathogenic micro-organism and to reduce the hygienic risk of organic wastes used as fertilizers. The hygienic risk can be minimized and the contamination of the environment can be avoided or reduced. It seems to be possible to produce a fertilizer with high quality in consideration of hygienic aspects by composting of organic wastes using tested variants.

Conclusion

The enormous amount of organic wastes, such as pig faeces, from intensive animal husbandry and meat production in China need a biotechnological treatment which guarantees the microbiological safety of produced fertilizers. Land application of raw manure also results in contamination of agricultural runoff and water supplies. Contaminated drinking water has the potential to cause extensive outbreaks. Salmonella is relatively persistent in soil compared to other pathogens (HOLLEY 2003) He noticed that proper composting of manure can yield safe

fertilizer and is obviously important for its use on food crops, but may also be important for forage crops to reduce levels of pathogens (*E. coli* O157 : H7) in livestock. The results of our experiments show that composting of pig faeces with corn stalks is a possible biotechnological treatment to recycle organic wastes, in consideration of hygienic safety, and to use and export nutrients to other regions. It should be possible to eliminate the major bacterial pathogens from pig faeces by composting in these variants. Our results for detection of test organism "W775" and the temperature profiles in different positions of rotting boxes show that there is an elimination of pathogens and therefore sanitation by composting. Our study demonstrates that all six variants of composting processes tested in our experiments were sufficient to eliminate "W775" providing temperatures as recommended. It seems to be a suitable treatment for organic wastes in order to produce a fertilizer with good hygienic quality and to minimize the risk for contamination of environment, therefore for safe feed, food and animal production. These fertilizers can be used to export nutrients from areas with high animal density to other regions, far away, with a minimized microbiological risk.

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Survival of Enteroaggregative Hemorrhagic *E. coli* (EAHEC) O104 : H4 and Monophasic Variant (O4, [5], 12 : I : -) of *Salmonella* Typhimurium in Organic Fertilizer such as Sewage Sludge, Slurry and Biogas Plant Effluents during Storage and Heat Inactivation

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Summary: This paper describes investigations about the tenacity of enteroaggregative hemorrhagic *Escherichia coli* (EAHEC) serotype O104 : H4 and monophasic variant O4, [5], 12 : I : -of *Salmonella enterica* serovar Typhimurium in organic fertilizers, such as sewage sludge, slurry and biogas plant effluents. In detail the investigations included experiments about the survival of both pathogens during storage of these fertilizers over month at 10℃ and experiments concerning their heat inactivation. The experiments are running and further results are still pending. Finally the studies should deliver information about the tenacity of tested bacteria, especially about the "new" enteroaggregative EAHEC serotype O104 : H4. The knowledge about the survival of pathogenic bacteria in organic fertilizer and their heat resistance can be used to estimate risks in use of these fertilizers on agricultural areas and optimize biotechnological treatments.

Introduction

Organic fertilizers, such as sewage sludge, slurry and biogas plant effluents, can contain pathogenic microorganism such as *Salmonella* and pathogenic *Escherichia coli* (*E. coli*) serotypes. By fertilizing of agricultural areas these pathogens can be spread into and through the environment into the food chain via plants, food of animal origin, and into drinking water and may cause human infections.

In Mai/June 2011 enteroaggregative hemorrhagic *E. coli* (EAHEC) serotype O104 : H4 caused an outbreak of severe foodborne infections with diarrhea, cases of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in Germany. In total 3842 cases of human infection with enteroaggregative hemorrhagic *E. coli* (EAHEC) O104 : H4 in Germany were recorded. It is an emerging *E. coli* pathotype that is endemic in Central Africa and has spread to Europe and Asia (Beutin et al, 2012). Based on sequence analysis the outbreak strain showed a 93% homology to a human EAEC-strain isolated in central Africa (BfR, 2011). The outbreak strain was probably transmitted by contaminated sprouted fenugreek seeds, imported from Egypt. This serotype is described as a recombination out of two pathogenic *E. coli* serotypes (EHEC and EAEC) in literature. EAHEC strains have evolved from enteroaggregative *E. coli* (EAEC) by uptake of a Shiga toxin 2a (Stx2a)-encoding bacteriophage. Except for Stx2a, no other EHEC-specific virulence markers including the locus of enterocyte effacement (eae-gene) are present in EAHEC strains. EAHEC O104 : H4 colonizes humans through aggregative adherence fimbrial pili encoded by the enteroaggregative *E. coli* plasmid. It shows a resistance to penicillin and cephalosporin and produces extended beta-lactamases (ESBL). Humans are the only known natural reservoir for EAHEC. In contrast, Shiga toxin-producing *E. coli* (STEC) and EHEC are associated with animals as natural hosts (Beutin et al, 2012). Serotypes of stx2-positive/negative EHEC O104 : H4 occurred sporadically in humans and were not detected in food before the outbreak. In general other serotypes of STEC/VTEC were frequently detected in food during the last years. Different serotypes of EHEC were detected in animals and the environment, but the outbreak strain never was detected. The reservoir seems to be humans, so the reproduction takes place in humans. By excretion over feces it can get into the environment, such as wastewater (sewage water) (BfR, 2011). Asymptomatic and symptomatic human excretors could be responsible for spreading of the outbreak strain into the food chain. During the outbreak, person-to-person transmission can also play a role. There is just small knowledge about the tenacity of the EAHEC serotype O104 : H4 in the environment. In general, EHEC have a relatively high tenacity against drying up, freezing or acidification, so that they are able to survive in the environment (soil, water and feces) over weeks and month.

Over the last years infections with monophasic strains of *Salmonella enterica* appeared and increased in Europe.

Hopkins et al noted a marked increase in the prevalence of *S. enterica* serovar 4,[5],12:I:- with resistance to ampicillin, streptomycin, sulphonamides and tetracyclines (R-type ASSuT) in food-borne infections and in pigs/pig meat in several European countries in the last ten years. This serovar was among the top 10 most common serovars isolated from both pigs and pig meat in the EU in 2006. Cases of infection have reportedly been severe, with a 70% hospitalization rate during an outbreak in New York City in 1998 (Hopkins et al, 2010). The Salmonella enterica serovar 4,[5],12 : I : - is characterized as a monophasic variant of *S. enterica* serovar Typhimurium (Echeita et al, 1999; Hauser et al 2010).

Pathogenic *E. coli* such as EHEC, EAEC and EAHEC strains and *Salmonella* are still very important (zoonotic) pathogens and responsible for high numbers of foodborne diseases all over the world. Especially the severe outbreak with EAHEC serotype O104 : H4 in Germany demonstrates the importance of these pathogens. During the last 15 years, monophasic serotypes of *Salmonella* emerged in Europe. In general *Salmonella* and *E. coli* have a relatively high tenacity, so they are able to survive over month in the environment and can be spread into the food chain and cause human infections.

The objectives were to determine the heat resistance and the survival of enteroaggregative hemorrhagic *Escherichia coli* (EAHEC) serotype O104 : H4 and monophasic variant (O4,[5],12 : I : -) of *Salmonella enterica* serovar Typhimurium in organic fertilizers such as slurry, sewage sludge and biogas plant effluents during storage over month. The challenge was to determine the tenacity of tested bacteria in these organic fertilizers. The influences of storage duration on the survival of these pathogens and sufficient temperatures for their inactivation should be examined in the experiments.

Material and methods

The studies were divided into two different experiments. On the one hand, the survival of tested bacteria in different organic fertilizers during storage over six month, and on the other hand, experiments about the heat inactivation of these pathogens were carried out.

In the storage experiment the organic fertilizers (sewage sludge, biogas plant effluents, and slurry) were contaminated by inoculation with overnight cultures of tested bacteria (EAHEC serotype O104 : H4, monophasic variant O4,[5],12 : I : - of *Salmonella enterica* serovar Typhimurium). Over six month, the contaminated samples were stored at 10℃ and microbiological examinations were carried out in the beginning, after two weeks and every month until the end of experiment. In compare, suspension (from overnight cultures) of each tested bacteria were stored at 4℃ and examined on same dates.

To determine the influences of temperature on the survival of these pathogens, contaminated samples were heated at different temperatures and durations in water bath or autoclave. The tested temperatures and the duration of the treatment were chose in consideration to the expected tenacity of the pathogens. As pathogens we choose EAHEC O104 : H4, monophasic O4,[5],12 : I : - of *S.* Typhimurium and a strain of *Escherichia coli* (DSM strain 682). Sewage sludge and slurry were used as substrates in the experiments.

Samples were stored at 4℃ or 10℃ until microbiological examination in the laboratory. For quantitative detection of EAHEC O104 : H4 resp. *S.* Typhimurium resp. *E. coli* 20 g (resp. ml) of each sample were weight in into 180 ml of sterile sodium chloride solution (0,9%), shaked and ten folded diluted. For each dilution three parallel tubes with BRILA-broth resp. buffered peptone water resp. Mac Conkey-broth were inoculated and incubated for 24 h (48 h) at 37℃. After incubation, the BRILA-broth was streaked out onto CHROM-agar STEC O104 : H4 and incubated for 24 – 48 h at 37℃. Positive colonies appear as malve colonies with a clear background colour of the CHROM-agar STEC O104 : H4. From pre-enrichment in buffered peptone water 0,1ml was inoculated in selective enrichment broth Rappaport-Vassiliadis (RV-broth) and incubated for 24 h at 42℃. After incubation the RV-broth was streaked out onto XLD- (Xylose-Lysine-Desoxycholate) and BPLS (Brilliantgreen-Phenolred-Lactose-Saccharose)-agar and incubated for 24 h at 37℃. Suspicious colonies were streaked out onto TSA-agar, incubated for 24 h at 37℃, to receive pure colonies. These colonies were confirmed as *Salmonella* O4, [5], 12 by object slide agglutination with *Salmonella* specific test-sera. From Mac Conkey-broth dilutions with positive tubes (colour changed from purple to yellow) were streaked out on Mac Conkey-agar to confirm the colonies as *E. coli*. The numbers of EAHEC O104 : H4, *Salmonella* and *E. coli* were quantified using the most probable number-method (mpn-method).

Results and discussion

In the beginning of the experiment microbiological examinations of the organic fertilizers and the contaminated samples were carried out. The bacterial counts for enteroaggregative hemorrhagic *E. coli* (EAHEC) serotype O104 : H4 resp. monophasic variant of *S. enterica* serovar Typhimurium ranged from 4.3×10^7 cfu/g to 4.3×10^8 cfu/g in contaminated sewage sludge, slurry and biogas effluents. Fig. 1 shows the first results of storage experiments, in detail the bacterial count for

EAHEC in contaminated (inoculated) sewage sludge, slurry and biogas effluents in compare to EAHEC suspension (stored at 4℃ as control sample) in the beginning, after two, four and eight weeks of storage at 10℃.

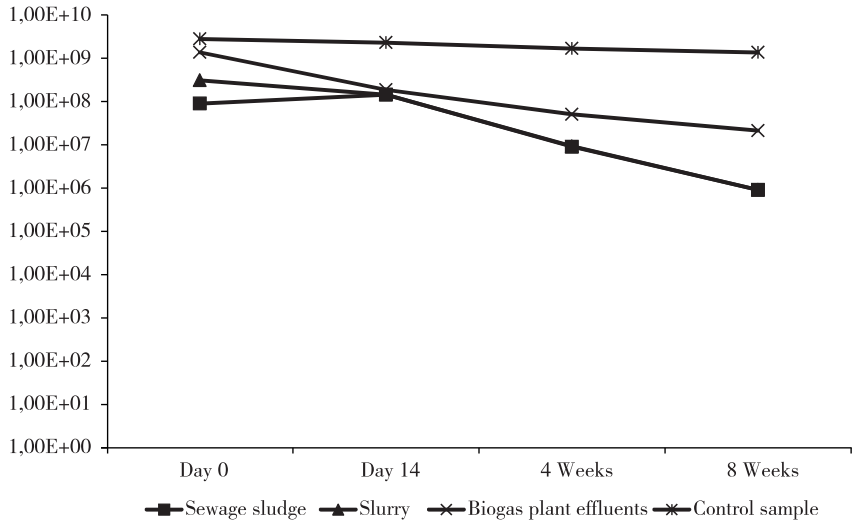

Fig. 1 Detection of EAHEC serotype O104 : H4 in sewage sludge, slurry, biogas plant effluents and during storage at 10℃ in compare to EAHEC suspension (control sample), stored at 4℃, on day 0, after 14 days, 4 weeks and 8 weeks.

The experiments (storage and heat inactivation) are running and further results are still pending.

Conclusions

Finally the studies should deliver information about the tenacity of tested bacteria, especially about the "new" enteroaggregative EAHEC serotype O104 : H4. The knowledge about the survival of pathogenic bacteria in organic fertilizer and their heat resistance can be used to estimate risks in use of these fertilizers on agricultural areas and optimize biotechnological treatments.

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Accumulation of Copper and Zinc in Fermentation Beddings of Swine Buildings

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Summary: Two typical pig farms with pig-on-litter system in Zhejiang Province was selected and monitored to discover heavy metal accumulation in litter. The litters in different buildings were sampled, and concentrations of total nitrogen (TN), total phosphorus (TP), copper (Cu) and zinc (Zn) were analyzed in this study. The results showed that the accumulations of Cu and Zn were affected significantly by the age of litters, Cu and Zn concentrations in feeds for different pig grow stages, and breeding density. The litter age was found to be the most important factor. The concentrations of Cu and Zn in litters of swine buildings with an age longer than two years were higher than the thresholds of the Chinese national standard. The TN and TP concentrations in litter were lower than that in the manure. The litter should be tested and treated before being used as fertilizer.

Introduction

Large quantities of animal wastes have been produced by farms as the livestock industry has grown rapidly, such as wastewater and manure, and disposal the wastes are difficult. Treating such waste is a huge and growing issue for pig producers worldwide as consumers are more concerned about animal welfare, environmental impact and meat quality in pig production systems. Pig farmers have to put great effort into animal waste treatment. To rectify this problem, the pig-on-litter system was developed initially in Japan as a zero-discharge treatment method, and different from a litter based straw system, microbial products were specially developed to accelerate the composting process. The deep-litter fermentation system is cheaper to establish and is perceived as being cost-effective and low-discharge, and it is not only beneficial for good animal production, but also for the welfare of the animal and the environment compared with conventional housing systems (Rebecca S, 2007). There has been a rapid increase in the use of low-cost, deep-litter swine housing systems in recent years in China.

The litter-bed pig house was made up of a conventional concrete floor where pigs can eat and rest, and a litter-bed area covered with a bedding material of a mixture of sawdust, rice hulls etc. to raise pigs and treat pig waste. Pig waste was thus treated in situ, with the manure-litter mix remaining in the pen before being used as a compost/fertilizer. However, there is a risk of heavy metals residue in litter due to the high level of heavy metals in pig feces and urine. Concentrations of heavy metals in litter are influenced by feeding, animal type, litter age, etc. And the bedding is always used for two to three years before they are removed for compost/fertilizer in China, The heavy metals in litters may posea risk to animal and human health through the litter-soil-food chain.

Research has shown that the average daily body weight gain, feed conversion ratio and survival rate of piglets raised in deep-litter pig pens were equal or superior to those raised in conventional concrete floor pig houses (Morrison, 2003; Sheng, 2009; Correa, 2009) as well as gaseous (ammonia, odor, et al) emissions from the deep-litter system (Wang, 2010). However, there isunavailable scientific literature regarding the impact of the deep litter fermentation system on the accumulation of nitrogen (N), phosphorus (P), copper (Cu) and zinc (Zn) in litter of fermentation bedding.

Two commercial swine farms with a deep-litter fermentation system were monitored in Zhejiang Province, China. The objective of this study was to evaluate the risk of the heavy metals accumulation in swine litter and the quality of the litter used as fertilizer.

Material and methods

Swine farms and houses

Two typical pig farms with pig-on-litter system in Zhejiang Province were selected and monitored. Farm A located in Lishui City, Zhejiang Province. The swine farm adopted an fermentation system in early 2009. Fourswine buildings with natural ventilation were chosen for this monitoring study. There were windows in the two long walls for ventilation and a concrete walkway in the piglets

and Grow-Finish buildings. Ad libitum feed and water was provided in fourthese buildings. The pit was 1 m high and was filled with the mixture of sawdust and rice hulls at the ratio 6 : 4 to a depth of 80 cm, and bacteria (Luodong enzymes) was added to aid fermentation. Litter was mechanically turned over weekly, and the new bedding materials and bacteria were added while turning the litter.

Farm B located in Jiangshan City, Zhejiang Province. The deep litter system was introduced in early 2008, and it was put in use in April, 2008. The same as farm A, it has long window on the wall for ventilation. The depth of the pit in piglets house is 40 cm, while the depth of the pool in growing houses is 80 cm. The ratio of sawdust and hulls which formed the beds is 4 to 6. The beds were turned over to the bottom every 7 to 10 days mechanically (Table 1).

Table 1 Description of the litter bedding in the swine buildings

Farm	Phase	Site	Start time	Volume of bedding (L × W × H, m^3)	Pig weight (kg)	Breeding density (m^2/ pig)
A	Piglets(1)	A1-1	2009.1	5.5 × 3.4 × 0.8	8 – 30	0.499
		A1-2	2009.3	5.5 × 3.4 × 0.8	8 – 30	0.499
	Grow-Finish(2)	A2-1	2009.6	11.3 × 5 × 0.8	30 – 110	1.130
		A2-2	2009.8	11.3 × 5 × 0.8	30 – 110	1.130
B	Piglets(1)	B1-1	2008.4	10 × 8 × 0.4	20 – 75	0.400
		B1-2	2009.3	10 × 8 × 0.4	20 – 75	0.400
		B1-3	2010.1	5.5 × 6 × 0.4	20 – 75	0.440
	Grow-Finish(2)	B2-1	2008.8	11.5 × 8 × 0.8	75 – 110	1.051
		B2-2	2009.7	15.3 × 8 × 0.8	75 – 110	1.399
		B2-3	2010.5	15.3 × 8 × 0.8	75 – 110	1.399

Table 2 Mean Concentrations of copper and zinc in the feed (mg/kg)

	Farm A		Farm B	
	Piglets	Grow-Finish	Piglets	Grow-Finish
Cu	190.9	131.8	174.7	112.4
Zn	266.8	141.5	307.1	166.9

Sample collection

The samples were collected in July 2010, December 2010, April 2011 and August 2012. Three sampling locations were selected in feeding area, resting and playing area, and discharging area respectively, the three sampling locations were chosen randomly in the manure bedding of sows building, the fresh samples were sampled at depth of 30 and 60 cm under the surface of the three locations in piglets and Grow-Finish buildings, and in the six monitoring buildings. Before the turning day, 250 g samples of litter were collected separately in the three locations and mixed, then taken back to the laboratory, sealed and stored in a refrigerator, samples were then mixed completely, and reduced by quartering to about 100 g. Then, the sampleswere crushed and sifted through a 1 mm sieve. Processed samples were stored in plastic bag. Sawdust and feed samples were also collected, processed and analyzed using the same method simultaneously for the determination of Cu, Zn, N, P.

Heavy Metal Concentration Analysis

The samples were treated and analyzed in the Key Open Laboratory on Ecological Agricultural Environmental Engineering of Chinese Agriculture Ministry. Total nitrogen was analyzed by sulphuric acid digestion followed by the micro-Kjeldahl distillation method, and total phosphate was analyzed by acid digestion and sodium bicarbonate extraction, respectively, followed by the molybdenum blue method, UV-Vis spectrophotometer.

Copper and Zn in the litter and feed were measured by the flame atomic absorption spectrometry method after acid digestion (Zeng-YeiHseu, 2004). The measurements were made under operating conditions for the most sensitive absorption lines of individual elements, i.e. Cu and Zn at 324.8 nm and 213.9 nm, respectively with a hollow cathode lamp current at 10 mA and a slit width of 0.7 nm.

Statistical Analysis

Differences among means were tested using the ANOVA procedure (SAS Version 8.2 software, SAS Institute Inc., Copyright, USA). A probability level of 0.01 was used to determine significance for each statistical test. Correlation between the concentrations of Cu and Zn in litter and the service time of litter was explored through regression analysis.

Results and discussion

Cu and Zn concentrations in Litter

The concentrations of Cu and Zn are shown in Fig. 1. Regression analysis were conducted to study the effect of the litter age, breeding density and copper and zinc concentrations in feed on the accumulation of Cu and Zn in litter. As shown in Table 3, the accumulations of Cu and Zn in litter were affected by the litter age, breeding density, copper and zinc concentrations in feed, the addition of new materials and the litter depth.

Table 3 Regression analysis results of the accumulation of Cu and Zn against influencing factors

Variable	Cu(R^2 = 0.87)			Zn(R^2 = 0.82)		
	Coef.	P	StdCoef.	Coef.	P	StdCoef.
Intercept	-287.5	0.0013	82.67	-309.5	0.1994	236.7
Cu (Zn) in feed (mg/kg)	1.699	<0.0001	0.3243	1.901	<0.0048	0.6319
Breeding density(m^2/ pig)	124.1	0.0096	45.51	14.90	0.9034	122.0
Litter age (month)	13.06	<0.0001	1.692	22.48	<0.0001	3.008

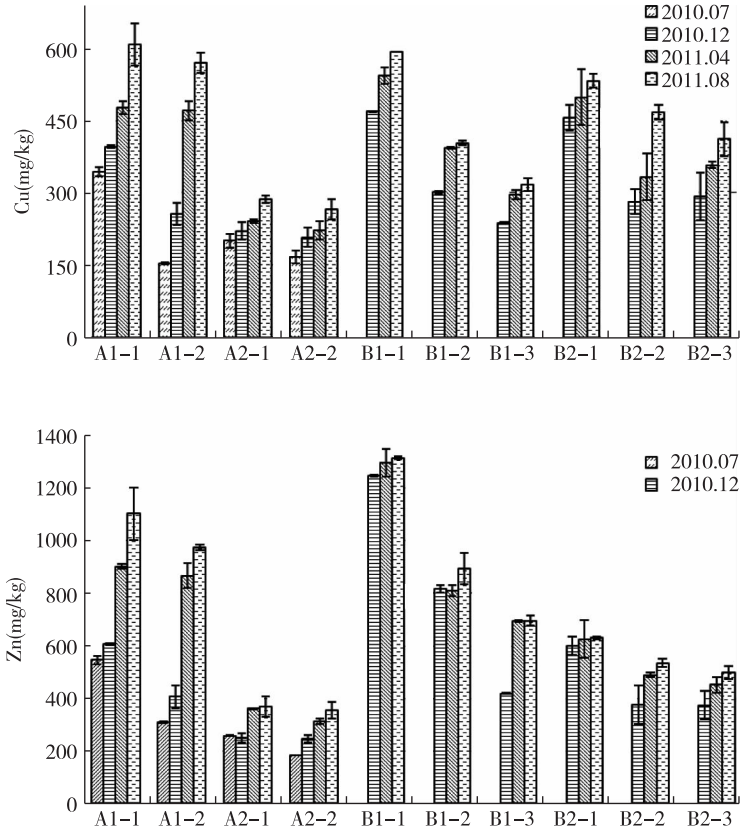

Fig. 1 Copper and zinc concentrations in litter of monitoring houses

Effect of litter age on the accumulation of Cu and Zn

The concentrations of Cu and Zn increased, as the litter age increased. Room A1-1 and A1-2 in Farm A had the same breeding density, the same management, and the pigs in the two rooms had the same feed and similar weight, thus the two rooms were taken together for analysis. There was a similar pattern between room A2-1 and A2-2. The relationships between Cu and Zn concentrations and the litter age in the piglets and Grow-Finish buildings were determined through linear regression, and the regression equations were established. The regression analysis (Table 4) showed that accumulation of Cu and Zn in litter of piglets rooms was positively correlated with the litter age ($P < 0.01$).

Table 4 Regressions between Cu and Zn concentrations and the litter age

Pig grow stages		Regression equation	R^2
Farm A Piglets	Cu	$Y = 27.8X - 249 (X \geqslant 16)$	0.89
	Zn	$Y = 51.9X - 516 (X \geqslant 16)$	0.90
Farm A Grow-Finish	Cu	$Y = 6.71X + 100 (X \geqslant 11)$	0.95
	Zn	$Y = 12.1X + 65.0 (X \geqslant 11)$	0.88
Farm B Piglets	Cu	$Y = 11.2X + 115 (X \geqslant 11)$	0.91
	Zn	$Y = 29.6X + 170 (X \geqslant 11)$	0.94
Farm B Grow-Finish	Cu	$Y = 7.66X + 260 (X \geqslant 7)$	0.90
	Zn	$Y = 9.34X + 318 (X \geqslant 7)$	0.88

Y: concentration of Cu or Zn, mg/kg; X: the litter age, months

It indicated that the concentrations of Cu and Zn increased as the litter age increased ($P < 0.01$). The concentration of Cu and Zn was directly correlated to the litter age in Grow-Finish rooms ($P < 0.01$), and significant correlations ($P < 0.01$) between Zn

concentration and service time of litter was observed at Grow-Finish rooms.

The relationships between Cu and Zn concentrations and the litter age in the piglets and Grow-Finish buildings in Farm B were determined through linear regression, and the regression equations were established. There were significant correlation between the concentrations of Cu and Zn and the litter age in piglets rooms in Farm B ($P < 0.01$). The Cu and Zn concentrations in litter of Grow-Finish rooms correlated with the service time positively ($P < 0.01$).

Effect of Cu and Zn concentrations in feed

The Cu and Zn concentration in feed varies among pig age andfarm, especially feed for piglets has higher concentrations of them (Table 2), the Cu and Zn in the feed were the source of the Cu and Zn in litter. As shown in Table 3, the concentrations of Cu and Zn in litter were especiallyaffected by the Cu ($P < 0.0001$) and Zn ($P < 0.0048$) concentrations in feed. Research at North Carolina State University indicated that concentrations of Cu and Zn in swine waste could be reduced through modifications of the diet (Teira-Esmatges, 2003).

Effectiveness and applicability of litter used directly as organic fertilizer

Over 73% of the soil was represented as acidified (< pH 6.5) except for the coastal plain in Zhejiang Province (Bian, 2009). Technology Code for land application rates of livestock and poultry manure (GB/T 25246 – 2010) are shown in Table 5. Therefore, the concentrations of Cu in litter from all buildings with longer than 1.5 years service time were higher than the limitation for use as vegetables fertilizer, and the concentrations of Cu in litter used one year in piglets and Growing-Finishing buildings had been already higher than the limitation for rice fertilizer, and the concentrations of Cu in litter used two years in piglets were higher than the limitations for dry crop fertilizer. The concentrations of Cu in litter with longer than two years in piglets were higher than the limitation for fruit fertilizer as well. The concentrations of Zn in the litter used 1.5 years in A1-1 were higher than the limitation for use as vegetable fertilizer, and the concentrations of Zn in the litter of piglets older than two years were higher than the limitations for rice fertilizer.

Table 5 Standards of Cu and Zn in animal manure (mg/kg, dry weight)

Plant	Cu			Zn		
	pH < 6.5	6.5 – 7.5	pH > 7.5	pH < 6.5	6.5 – 7.5	pH > 7.5
Vegetable	85	170	170	500	700	900
Rice	150	300	300	900	1200	1500
Dry crop	300	600	600	2000	2700	2700
Fruit	400	800	800	2000	2700	3400

Table 6 Mean TN and TP concentrations (Mean ± SD) in the litter (g/kg)

	TN			TP(P_2O_5)		
	2010.12	2011.4	2011.8	2010.12	2011.4	2011.8
A1-1	11.7 ± 0.9	13.7 ± 0.6	14.0 ± 0.6	14.7 ± 2.6	23.9 ± 0.7	24.6 ± 0.4
A1-2	13.3 ± 0.4	14.5 ± 0.3	14.8 ± 0.3	19.7 ± 1.7	23.8 ± 0.5	23.8 ± 1.3
A2-1	11.8 ± 0.3	14.1 ± 0.2	13.3 ± 0.6	18.2 ± 2.0	26.3 ± 0.7	25.2 ± 0.7
A2-2	13.7 ± 0.4	14.6 ± 0.8	13.8 ± 0.1	17.9 ± 0.4	22.7 ± 1.4	23.0 ± 1.5
B1-1	11.8 ± 0.7	11.8 ± 0.1	12.7 ± 0.2	19.9 ± 0.7	21.3 ± 0.1	22.6 ± 0.53
B1-2	9.8 ± 0.1	10.7 ± 0.2	11.9 ± 0.4	16.6 ± 0.2	17.9 ± 0.5	18.9 ± 0.82
B1-3	7.9 ± 0.1	8.6 ± 0.2	9.4 ± 0.1	12.6 ± 0.2	14.2 ± 0.3	15.8 ± 0.26
B2-1	9.3 ± 1.0	10.3 ± 0.7	10.5 ± 0.9	16.0 ± 0.2	16.1 ± 1.9	17.3 ± 1.7
B2-2	8.7 ± 0.5	10.1 ± 0.9	10.4 ± 0.3	16.2 ± 0.8	18.6 ± 2.0	18.2 ± 4.6
B2-3	7.7 ± 0.6	8.1 ± 1.4	8.2 ± 0.8	12.4 ± 2.3	14.9 ± 1.8	15.9 ± 2.3

The overall finding was that the litter from fermentation bedding in all monitoring buildings could not be directly used as fertilizer after two to three years service. The Cu and Zn contamination of the litter may be reduced if diets are formulated to provide Cu and Zn exactly matching the requirement of the animals. To guarantee the preservation of the environment and the wide applied of cost-effective and low-discharge pig-on litter systems, control of Cu and Zn concentrations in swine feed additives and litter management are urgently needed.

The concentrations of TN and TP (P_2O_5) in December 2010, April 2011 and August 2011 are shown in Table 6. The concentrations of N and P in litter were elevated from December 2010 to August 2011. The mean concentrations of TN and TP were 10.7 and 16.5 g/kg in December 2010, 12.1 and 20.9 g/kg in August 2011, respectively. According to Europe Eco-Label Standards

applicable to composts, application rates shall specify not more than: 17 g/m^2 N, and 6 g/m^2 P_2O_5. The litter used as fertilizer should have no more than 15.9 t/a (December 2011), 14.5 t/a (April 2011), 14.1 t/a (August 2011).

Conclusions

The accumulation of Cu and Zn in litter was affected by the litter age, breeding density, copper and zinc concentrations in feed. The accumulation of Cu, Zn in litter increased significantly with the litter age increased. The Cu and Zn added into the feed was the origin of the metals in the litter. The concentrations of Cu and Zn in nursery litter were highest due to the greater concentrations of Cu and Zn in the feed. The litter used as organic fertilizer should be restricted and pretreated while it is removed from swine buildings with longer than 2 years service time, especially while it applied on vegetable fields.

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Impact of Ground Water Arsenic on Fish Farming in the Terai Regions of Nepal

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Abstract: Fish farming is an important component of Nepalese farming system and today it is becoming one of the important occupations in the Terai region of Nepal. Fish farming is vital and it is the source of meat and household income. Gradually fish farming is changing its nature from subsistence form to commercial stage basically in the Terai regions of Nepal. Lots of potentials and challenges are prevalent in Nepalese fish farming. Higher amount of arsenic is found in ground water of Terai regions in Nepal. This study covers the major concerns over the glimpse of fish farming focusing the current status of arsenic in ground water and its impact on fish farming. Review of literatures and information are compiled to meet the objective. Leaching of arsenic from rocks has been the main source contamination of water in Nepal. Higher level of arsenic is found in Nawalparasi, Rupandehi, Kapilbastu and other districts of Terai Region. The arsenic present in the ground water is causing various problems associated with health of fish as well as the health of people. Higher level of arsenic is causing problems in proper management of farms as well. Various preventive and mitigation majors should be employed to reduce the risk of the effect of arsenic for sustainable and higher fish production. There is an urgent need for a detailed and scientific study to be carried out in this arena.

Key words: Terai region, arsenic, fish farming

Precision Livestock Farming（PLF）

The Use of Pedometers as Supervision Tools for Cows and Mares in the Prepartal Period

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Summary: To assess the practical use of pedometers as supervision tools in the prepartal period, IceQube® -and ALT-sensors were tested on mares and cows. Animals were fitted with one pedometer type on the left and the other one on the right front leg, and in case of 7 mares also with an IceQube® on a neck collar. Motion activity, lying times and lying bouts were measured in the time period 10 days ante partum. Function and animal acceptance of the devices, and the informative value and the general use of the assessed data to predict the date of parturition, were investigated. Therefore, deviations in the animals' behavior were statistically determined by estimating Least-Squares-Means (LSM) for hours with 4 records each hour, and differences of these LSM between days ante partum for each respective hour ($\alpha = 0.05$). The LSM-differences for motion activity in horses showed a highly significant increase 1 – 2 hours ante partum. Cows also showed a significant increase in motion activity during the last prepartal hours; however, with a higher variability in reference data. Therefore, the parameter is less predictive for cattle. Lying time, both for mares and cows, showed to be of less informative value. In mares, movements of the head and the neck, measured by the device on the neck collar, increased significantly in the last prepartal hours. We suggest that this parameter could have a high predictive value. In both species, the number of lying bouts increased significantly prior to parturition. We propose a high potential of pedometers' use as supervision tools in the prepartal period of mares and cows for both examined pedometer types. However, technic and software have to be adapted and improved.

Introduction

Both cows and mares partly show wide variations in their gestation length. Those deviations are dependent on multiple factors, such as the breed, the fetal sex, the photoperiod and the mother's parity, which are described detailed by Foote (1981) and Satué et al. (2011) e. g.. Periods of 315 – 388 days post conceptionem in mares (Davies Morel et al., 2002) and 259 – 294 days in cows (Jafar et al., 1950), usually results in vital progeny. The calculated date of parturition can only serve as an approximate assessment value; however, with regard to management and personnel costs, an exact prediction of this date is desirable. The aim of this study was to evaluate the practical application of two types of pedometers for the supervision of cows and mares in their prepartal period. Technical function, animal acceptance, informative value of the assessed data, and the general use of pedometers as predictive instruments, were investigated.

Material and methods

IceQube® -(IceRobotics Ltd. Edinburgh, UK) and ALT-pedometers (Engineers Holz Falkenhagen, Germany) were tested on 9 mares, and 12 cows and heifers. Animals were fitted on each front leg with one device (IceQube® right, ALT left), and in case of 7 mares also on a neck collar (IceQube®). Measurements started 10 days before the calculated day of birth with a measure interval of 15 minutes. Partly, the measuring periods differed from the 10-day target because of higher variations in gestational length. Statistical analysis was performed with the software package SAS 9.2 (SAS Institute Inc. Cary, USA). Using the MIXED procedure, LSM were estimated for hours with 4 records each hour. Through a significance test of the LSM between the days ante partum for each respective hour, deviations in the animals' activity and lying behavior were assessed on the day of birth, especially in the last hours prior to parturition. For statistical analysis the prepartal behavior of investigated animals was expressed by the parameters motion activity (MA), lying time (LT), lying bouts (LB), and indirect neck activity (iNA) additionally for those mares fitted with a neck sensor. MA included in addition to moving behavior every measureable motion impulse. Both devices summarized the assessed data as a motion index. Also the LT-marker was expressed as index data. For LT, IceQube® -data was adapted to the ALT-index with in maximum 60 records per 15 minutes. LB was only measured by the

IceQube® -devices. All parameters were analyzed firstly for the group, and as a validating step, also for every single animal.

Results

Mares: Mean gestation length of the studied mares was 337.3 ± 8.55 days, in a range of 326 – 356 days. All equine births ran without any difficulties and resulted in vital foals. Because of the flexible IceQube® -and the new constructed ALT-fastening systems (Saddlery Weidner Halle, Germany), we could not detect any pressure or chafe marks or any disorder in animal behavior. The LSM-differences assessed a highly significant increase of the mares' MA 1 – 2 hours ante partum in comparison to the activity in the same hours on each of the 10 days before (Fig. 1). The daily times on pasture or paddock differed significantly from the reference level, and were therefore analyzed separately. The correlation between the instruments' data for MA was calculated with r = 0.51; respectively with r = 0.73 including the pasture and paddock times. The parameters LT and LB of the mares were only reasonable analyzable during stable times. LT increased significantly on the day of parturition with high individual variations, but usually too close to parturition. The data correlation between the pedometer types for LT was r = 0.71. The IceQube® -measurements from the neck collars recognized a permanent lying of the devices. These sensors were not able to generate useful data for the MA-marker; however, with increasing activity of the mares' head and neck (iNA), the apparent lying bout was dissolved, and the data dropped highly significant under the reference level. The analysis of LB showed a significant increase within the last hours ante partum.

Cattle: Heifers showed a little longer mean duration of pregnancy with 279.6 ± 0.94 days post conceptionem (278 – 281 days), compared to the cows with 276.6 ± 3.86 days (272 – 283 days). Calves were born healthy, but in few cases with complications during parturition. In congruence with the results of the mare data, cows and heifers showed a significant increase of MA during the last prepartal hours. However, those differences were also possible within the other days ante partum, because of a higher variability in reference data. For MA, the data correlated with r = 0.75 between devices. LT was not useable for predicting a near parturition of cows and heifers. ALT-and IceQube® -data for LT correlated with r = 0.86 in cattle. The animals' typical daily lying bouts began to fragment markedly before parturition, and thereupon the LB-marker increased significantly.

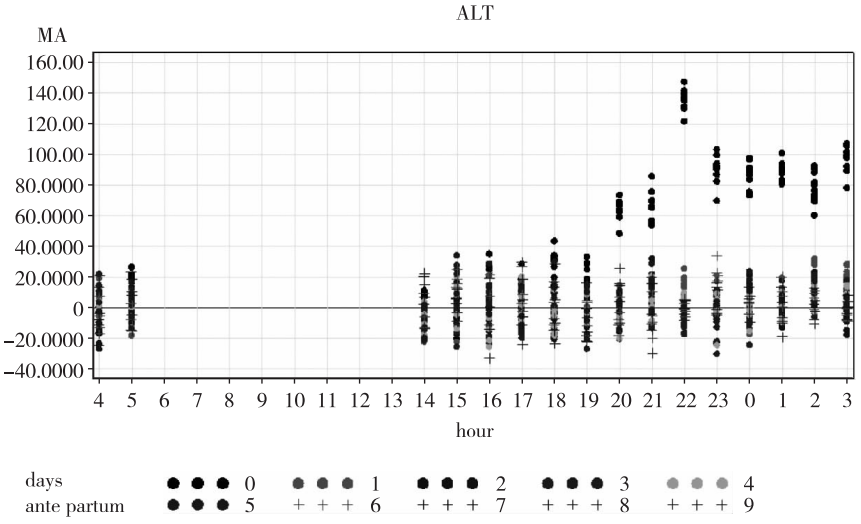

Fig. 1 Differences of LSM for motion activity (MA) in mares between days ante partum, and the day of parturition within the same hour (method: ALT, n = 9)

Discussion

All progeny were born healthy and within a physiological gestation period as reported by Jafar et al. (1950) and Davies Morel et al. (2002). However, on base of the small number of investigated animals, we cannot discuss an influence of parity, photoperiod, the fetal gender, or the breed, or any typical diurnal scatter of birth time. Although, all observed equine births happened at late evening or within the night; all bovine births also during the midday hours. This affirms the animals' high necessity of quietness during birth (Newcombe & Nout, 1998). The calculated phenotypic correlation reflects differences between ALT-and IceQube® - readings, which could be explained by different preferences in motion of one side of the body,

and by the aberrant sensibility of the sensors.

In mares, MA increased significantly 1 – 2 hours ante partum in comparison to the homogenous activity in the same hours on each of the 10 days before. The daily times on pasture or paddock differed markedly from the reference level of the stable times, and were therefore analyzed separately. Several mares showed a beginning increase in motion activity during these paddock or pasture times on the day of parturition or already on the day before. Therefore, these periods should not exclude completely. For the parameters LT and LB, only the data during stable times gave adequate information. LT was due to its increase too close to parturition, not useable as a predictive parameter. In the last prepartal hours, movements of the head and the neck (iNA) increased significantly. This could be explained by the colic like behavior under pain, where the mare looks with increasing intensity in abdominal direction. We suggest that this parameter could have a high predictive value.

In cattle, MA cannot be recommended as a predictive parameter, because of the high variability in the reference data. Results of data analysis of LT showed significant differences between the day of parturition and the other days before; however, the associated reference level was less homogeneous.

The sum of lying events per day and per animal showed an obvious decrease of daily total LT on the day of parturition, as described also by Raya et al. (2009) and Jensen (2012). With the decrease in daily total LT, the number of LB increased. The typical long daily lying bouts began to fragment markedly, as assessed before by Huzzey et al. (2005). In this study, prepartal behavioral changes were detected at the earliest within the last 4 days ante partum. In general, a period of 10 days should give enough space to collect data for reference. To avoid a frequently false alarm within the first days of research, we recommend a predictive system based on pedometer technique with the possibility to switch from an adaptive phase of data collection for reference data to an alert mode at day 4 ante partum.

Conclusions

In conclusion this study showed a high potential of pedometers' use as supervision tools in the prepartal period of mares and cows, if the correct parameters are used. Future investigations should focus on the definition of individual thresholds for the prepartal changes in animals' behavior that can be used in a prediction system. Further, pedometer technic and software have to be adapted. Particularly the high sensibility of the IceQube® s sensor should be adjustable. Concluding, on base of the assessed data, none of the tested pedometer types can be preferred. However, IceQube ® -sensors seemed to be more suitable for a further development. More research is required to improve the reliability of such birth detection systems.

Acknowledgement

The authors like to thank Istvan Gyongy and Robert Boyce of IceRobotics Ltd., UK for pedometer provision and informational support. We also thank all employees in Köllitsch and Graditz for their pleasant help during the practical research period.

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Development of an Early Alarm System for a Broiler House using Image Distribution Index Prediction

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Summary: Anomalous animal behavior and reduced growth rate are just a few signs that can indicate an undesired situation in a broiler house. It is important that problems such as diseases, technical malfunctioning in feeding and drinking lines and suboptimal management procedures are detected in an early stage to avoid harming the welfare or the production results of broilers. This paper introduces an automated method to detect problems in a broiler house using cameras and an image analysis software. Three top view cameras mounted in the ridge of a house at the height of 5 with a dimension of 19.8 by 63.5 meters continuously monitored the floor space below. Analysis software translated these images into animal distribution index in the house. The final objective was to develop a system that could report malfunctioning in a broiler house to the farmer in real-time. In an experiment with Ross 308 broilers, distribution index data were collected every 5 minutes in a commercial broiler house with 28000 animals. Based on the distribution index data, a linear real-time model was developed and tested to model the animal distribution index as a response to the light input. Using this model, an online prediction could be made on animal distribution index. By comparing the predicted values with the measurements in real-time, malfunctioning could be detected. Results showed that this method was able to report 95.24 per cent of events (20 out of 21) in real-time.

Introduction

According to FAO [1], during this decade, the expected annual world poultry growing is 2.8%. Therefore, intensive broiler-keeping is unavoidable and management in broiler houses is crucial. Technology offers a high potential for real time monitoring of livestock [2]. Applying technology in livestock production is the core idea of Precision Livestock Farming (PLF) [2] Continuous automated monitoring of agricultural animals can result in "early warning systems" that improve the management of (individual) animal needs at any time. Accordingly, employing a tool to monitor the broilers can help the farmer substantially to manage his house more efficiently [3].

Regarding the man-hours needed for a person to check a broiler house regularly, this is a cost-effective approach that can meet daily needs of broiler house caretakers. On average the monitoring tool used in this work, namely eYeNamic, could save 120 farmer inspection hours and reduced losses by 45 broilers during a growth period (42 days) in 5 commercial houses with an average of 28000 broilers.

Technology of monitoring broilers by image processing has already been practiced by many scientific researchers. Kristensen and Cornou [3] investigated possibility of detecting leg disorders in broiler chickens through analyzing deviations in activity level measured by image analysis. But what is missing in previous works is an algorithm that can report problems of the poultry house in real-time and can help the farmer to manage keeping his broilers more efficiently [4].

Objective of this paper was to describe a method for early warning of events in a commercial house using measuring the distribution index of broilers and a real time monitoring technique.

Material and methods

The eYeNamic system

The eYeNamic system is a useful tool used for livestock monitoring [5]. In the experiments of this work, this system was consisted of a setup with 3 top-view cameras distributed over the length of the house which was 63.5 meters long. Ientical Mobotix M24SEC22-D22 IP-cameras were installed in the ridge at the height of 5 meters and evenly distributed over the entire length of the house. Images were captured with a resolution of 1280 by 960 pixels and a 0.5 Hz frame rate in MxPEG format. Software was developed to measure the distribution index of animals, visible in the camera image, in real time and in practical conditions. Fig. 1 shows how the input images looked like when three cameras were used in the house in a surface of 19.8 by 63.5 meters with broilers at the age of 27 days.

Birds and housing

Broilers were kept in a commercial broiler house for a growing period of 42 days. Mean air temperature was set at 34℃ during day 1 while temperature was decreased gradually until 20℃ at the end. Light was switched on and off four times a day, so there were 4 light periods

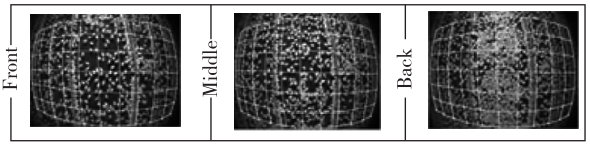

Fig. 1 Picture of the ground surface in a broiler house equipped with eYeNamic divided to 60 1 by1 meter zones in one camera's image

with a minimum light intensity of 5 lux and a maximum of 10 lux (when the light was on) for 5 hours and 4 dark periods (when the light was off). The start of the first light period was at 3AM.

Equipment and data collection

Images collected continuously from the eYeNamic system over a whole growth period (42 days) together with time and date labels were exported to CSV files. These files were analyzed in MATLAB in real-time. eYeNamic data were collected every 5 minutes resulting in 2880 hours of videos (5,184,000 images) in total.

In addition, a logbook was filled in by the farmer indicating the events happening in the house. This was taken as a reference to validate the algorithm. Fig. 2 show the process of data processing using eYeNamic monitor tool versus manual scoring of the events by farmer.

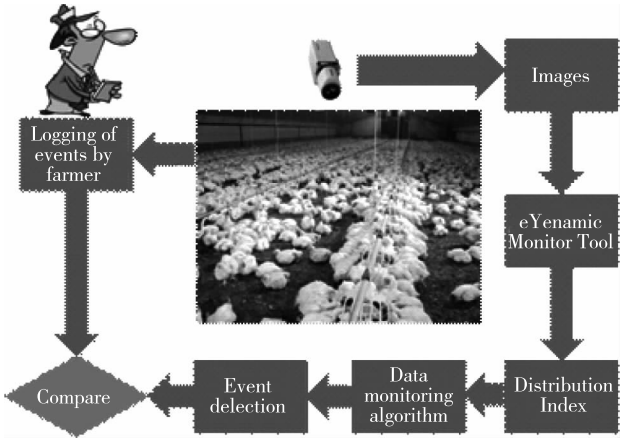

Fig. 2 Process of data processing using eYeNamic monitor tool versus manual scoring of the events by farmer

Distribution index calculation

To calculate the animal distribution index, the image captured by each camera was divided to 10×6 zones and the occupation density of broilers in each zone was considered after binarizing the image using histogram shape-based thresholding explained by Buyse J et al. [6].

Zone Occupation Density (ZOD) in zone (i,j) was calculated using equation 1.

$$ZOD_{i,j}(t) = \frac{\sum(x,y) \in z_{i,j} O(x,y,t)}{Z_s(i,j)} * 100 \qquad (1)$$

In the above equation, $O(x,y,t)$ is the occupation (foreground pixels in the binary image) of a zone (grids in Fig. 1) and Z_s is the size of the zone in pixels.

There were in total 180 zones (Fig. 1). For covering a total of 28000 birds, the average occupation rate of all zones from the different cameras was calculated using equation 2.

$$\overline{ZOD(t)} = \frac{\sum_C^C \sum_i^M \sum_j^N ZOD_{i,j}(t)}{C \times M \times N} \qquad (2)$$

In the above equation, M and N are the number of rows and columns of zones respectively and C is the number of cameras. Using $ZOD(i,j)(t)$ of each of the cameras The distribution index is calculated from the three matrices. All values are checked to see how many of them are out of the range of 20% from $\overline{ZOD(t)}$. Equation 3 shows how this calculation is performed ($\alpha = 0.2$ or 20%).

$$U_{i,j}(t) = \begin{cases} 1 & \text{if} \left| ZOD_{i,j}(t) - \overline{ZOD}(t) \right| < \alpha \times \overline{ZOD}(t) \\ 0 & \text{else} \end{cases} \qquad (3)$$

Finally, the distribution index is yielded by equation 4 ($\alpha = 0.2$).

$$UI(t) = \frac{\#\text{zones with} \left| ZOD_{i,j}(t) - \overline{ZOD}(t) \right| < \alpha \times \overline{ZOD}(t)}{C \times M \times N} \qquad (4)$$

Fig. 3 shows the distribution index of the observed farm during a full growth period of 42 days. In this figure a sequence of light and dark periods is magnified to illustrate the concept.

EDI (eYeNamic Data Interpretation) algorithm

Distribution index of broilers in a broiler house follows a rather standard trend during the growth period and increases over time rather linearly (Fig. 3). This trend can be affected by several factors including problems in feeding or drinking system and light intensity as shown in Fig. 3, thus analyzing the data can help to detect the events happening in the house. To detect these events, a model-based algorithm was developed.

Development of the adaptive real-time model

A linear real-time model [7] was developed and tested to model the distribution index of the birds as a response to the light input. Since distribution index varies linearly over time, this model is designed to predict the data of next light periods using the average slope of the previous periods. This model is simple, fast and implementable for real-time applications. Using this model, an online prediction could be made on the distribution index each time the light was switched on.

In case the measured values are deviating from the

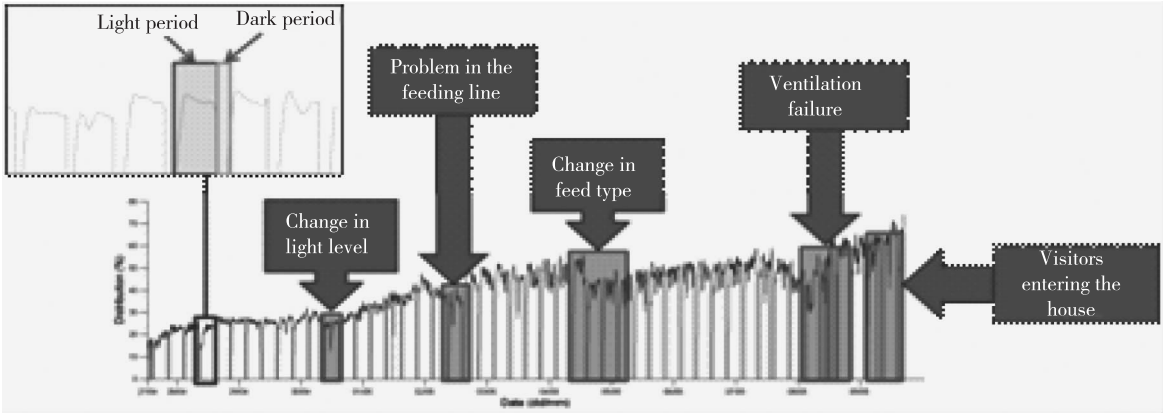

Fig. 3 Distribution index for the commercial farm in a full growth period of broilers; Occurrences are also shown

predicted standard values, an event might have happened in the house. As shown in Fig. 4, the predicted values are categorized based on deviation from the measured values (thin grey line) as follows: (1) thick bright grey line: less than 25% of negative or positive deviation from the measured values; this means the prediction is fulfilled. (2) thick dark grey line: more than 25% of negative or positive deviation from the measured values; this means the prediction has lost following the measured values. If the faulty (dark thick grey line) region continues for more than 15 minutes, an alarm will be generated.

The prediction model explained above works based on a moving window. The moving window is shifted for one light period each time and next light period data are predicted.

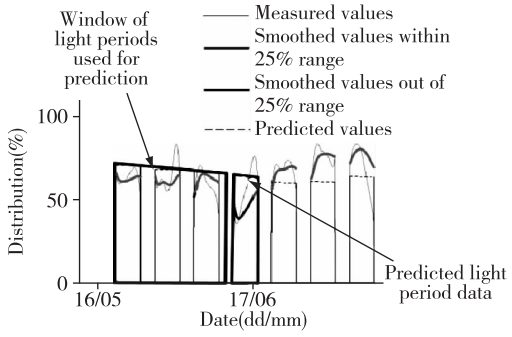

Fig. 4 Prediction window (consisting of three light periods) to predict the next light period data

Validation of the model

A logbook was also filled by the farmer. He filled in the logbook whenever he knew there was a problem, for instance with feeder lines or when he was observing an abnormal behavior of broilers. The events recorded by him were compared with the alarm regions (thick dark grey line in Fig. 4) generated by the algorithm. The results of the comparison will follow in the next section.

Results and discussion

In this paper an algorithm was described to detect occurrences in a broiler house using image interpretation and analyzing the distribution index of broilers in captured images. Fig. 5 demonstrates the predicted and the measured values that align to a precise extent. In this phase a linear real-time model was used. Subsequently, alarms generated by the algorithm were compared with the events logbook filled in by the farmer.

The results of applying the algorithm on the data of the commercial broiler farm for a complete fattening period are presented in Fig. 6. Taking events logbook of the farmer as reference, the EDI algorithm managed to successfully detect 20 (95.24%) out of 21 of the events happening in the house.

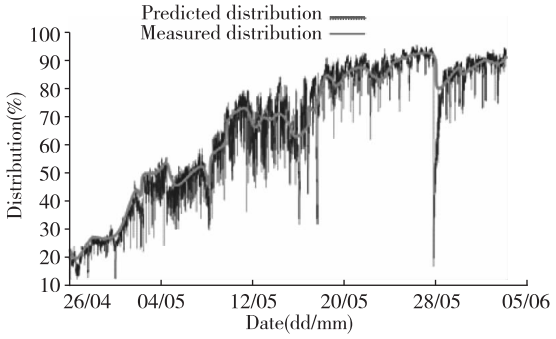

Fig. 5 Distribution index of the commercial farm over a growth period (42 days)

True positive cases using EDI were 20 (95.24%) vs. 13 (61.9%) using [8], False negative cases were 1 (4.76%) vs. 2 (9.5%) and False positive cases using EDI were 0 (0%) vs. 2 (28.6%).

Conclusion

A technique has been introduced that offers fully

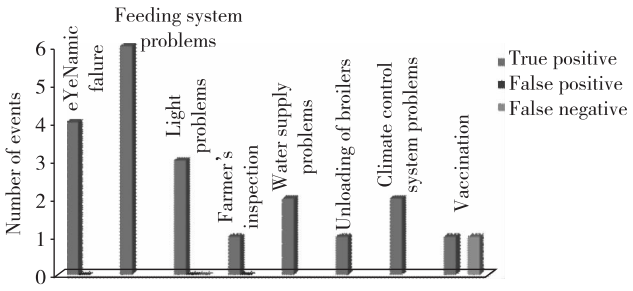

Fig. 6 Categorization of events and comparison of performance of EDI algorithm with the algorithm presented by [8]

automated identification of problems in a broiler house. This became possible by performing real-time camera vision-based monitoring based on top-view video processing and linear real-time prediction models. The results showed that by real time prediction of distribution index of broilers, it is possible to detect problems in feeding, drinking, heating and ventilation systems and vaccination effects. So far, the system is able to detect these problems with an accuracy of 95. 24% while no unwanted alarm was provoked. In conclusion, the introduced method is an important economic factor for the livestock sector since feed and water intake, health, welfare, performance and farm profitability are all variables that are important to be monitored.

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The Relationship Between Physiological Status and the Hatching Time in the Newly Hatched Brolier Chicks

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Summary: This study evaluated the influence of hatching time on physiological status of 40 newly hatched broiler chicks at the end of incubation. Four batches (300 eggs/ batch) of Ross 308 eggs were incubated in two small scale custom built incubators (Petersime NV). The hatching time of focal eggs was monitored using video cameras. Following the commercial practice of take-off' (when all chicks are removed from the machine once the majority of the chicks have hatched), focal chicks were assessed using the standard chick quality scoring method and physiological parameters: body weight, organ (heart, liver and stomach) weights and plasma corticosterone level. The time of take-off minus the individual's hatching time gave the duration each focal chick spent waiting in the machine. The focal chicks waited between 7.5 to 45.3 hours with the majority of chicks waiting in the machines for 20 to 30 hours after hatch. Positive association of heart weight with chick weight ($r = 0.6$, $P = 0.001$) and relative stomach weight with waiting time ($r = 0.6$, $P = 0.001$) were found. In addition, chick weight is significantly associated ($r = 0.9$) with egg weight ($P < 0.001$) and waiting time ($P = 0.02$). Furthermore, there was a positive correlation between plasma corticosterone levels and waiting time ($r = 0.6$, $P = 0.03$). Specifically, chicks that hatched early had higher corticosterone levels than chicks that hatched later which indicate that chicks might be more stressed the longer they waited. We conclude that it is important to shorten the hatch window in order to minimize the number of chicks that experience a long wait for take-off and hence heightened stress, which may be due to challenging environmental conditions or an extended period of feed and water deprivation.

Introduction

In a commercial hatchery, chicks hatch over a 24 – 48 hour period (Careghi et al., 2005). Therefore, the spread of hatch causes variability in the waiting time for chicks from emerging the shell until removal from the incubator when the majority of the chicks have hatched. Moreover, the newly hatched chick has to undergo several hatchery treatments and is then transported before being placed on the broiler farm. Therefore, the total time chicks are deprived of feed and water can be up to 72 hours (Willemsen et al., 2010). It has been proved that feed deprivation after hatch leads to dehydration (van de Ven et al., 2009), and impairs welfare and post hatch performance with respect to growth, immune system activation, digestive enzyme stimulation and organ development (Willemsen et al., 2010, Decuypere et al., 2001, Gonzales et al., 2003). Day-old chicks are the starting material to realise high-quality of a broiler flock at slaughter age(Tona et al., 2003). Furthermore, high-quality day-old chicks ensure greater survivability and better growth potential during the early stage (Christensen, 2009). However, there is no specific data showing the physical status and quality of newly hatch broiler within a wide spread of the hatch time. In this study, we identified the exact hatch time of focal eggs using video images and investigated the effects of waiting time on the physical status of newly hatched broiler chicks. Our result gives evidence of the importance of shortening the hatch window, and thereby decreasing the negative effects caused by long waiting time and optimising the chick quality.

Material and methods

Four batches of 2400 fertilized Ross 308 eggs were obtained from a local supplier (Henry Stewart & Co. Ltd, Lincolnshire, UK) and incubated in two small custom-built "BioStreamer" incubators (Petersime NV, Zulte, Belgium). Each incubator was able to set 300 eggs in 2 trays. Incubation conditions were continuously

monitored and controlled by the incubator controller pc (BIO-IRIS, Petersime TM). All incubation conditions (machine temperature, humidity, CO_2 concentration and ventilation rate) were identical in the two incubators. Eggs were candled at day 18 and those with evidence of a living embryo were transferred from the trays to the hatching baskets. Both machines were stopped after 512 h (21 days and 8 hours) of incubation.

Ten focal eggs out of 300 eggs of each incubator in each batch were individually labeled. During transfer the focal eggs were individually placed in special designed area (8 cm × 8 cm × 8 cm metallic mesh grid) of the top basket. The hatching time of individual focal eggs was determined using a digital color CCD camera VDC 413 (Inter M, Korea) which was attached to the internal ceiling of the incubator. The camera was combined with Milestone surveillance software and images were taken every second (1 fps) within 5 s when the camera was triggered every 5 min. Video decoders linked the camera to two PC's where the videos were stored for further analysis. The labeling of hatch time was based on seeing the chick just emerge from the egg and transferred to incubation time in hours. The waiting time of newly hatched chicks in the machine (hours) is calculated based on hatch time and take off time.

All the successfully hatched focal chicks were weighed and scored using a standard method (Tona et al., 2003). And then they were killed (dislocation of the neck) and blood samples were collected. Their organs (heart, liver and stomach) were dissected and weighed. Relative heart weight (RHW), liver weight (RLW) and stomach weight (RSW) were calculated by dividing organ weight by chick weight.

Blood samples were collected into heparin coated tubes and were centrifuged at 3,000 rpm for 10 min. The plasma was decanted into 1.5 ml tubes and frozen at −20℃ for corticosterone analysis. Plasma corticosterone (CORT) was measured using a commercially available double antibody RIA-kit (IDS Ltd, Boldon, England). All samples were run in the same assay in order to avoid inter-assay variability (Tona et al., 2003).

Data of the focal chicks from four batches were statistically analysed using SPSS (PASW statistics 18). All parameters were tested for normal distribution using a Shapiro-Wilk analysis. The non-parametric Kruskal-Walis test was used for the chick quality against batch and incubators. The differences in waiting time, chick measurement and CORT levels between batches (1to 4) and incubators (1 and 2) were examined in a General Linear Model (GLM). The relationship between parameters was determined using linear regression ($P < 0.05$).

Animal experiments were performed with ethics approval from the Royal Veterinary College Animal Ethics Committee.

Results and discussion

The spread of hatch and waiting time

There were 40 focal eggs which were successfully hatched. As there was no effect of batch and incubator on waiting time, the data of 40 focal chicks from four batches and two incubators was combined for analysis. The spread of waiting time in 40 focal chicks varied from 7.5 h to 45.3 h (Fig. 1) and the average of waiting time was 27.0 h ± 7.3 h. 50% chicks have to wait 20 to 30 hours until take off.

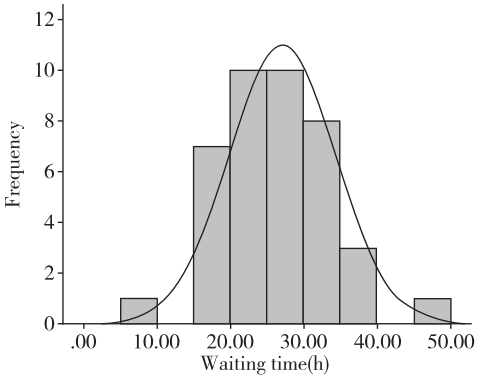

Fig. 1 The distribution of waiting time of 40 chicks

Waiting time and chick weight effects on chick quality and parameters

The average egg weights and chick weights were 66.90 g ± 4.90 g and 44.86 g ± 4.19 g, respectively. Chick weight is significantly associated ($r = 0.9$) with egg weight ($P < 0.001$) and waiting time ($P = 0.02$): chick weight = 0.778 × (egg weight) − 0.088 × (waiting time) − 4.805. Furthermore, Positive associations of heart weight with chick weight ($r = 0.6$, $P = 0.001$; Fig. 2) and relative stomach weight with waiting time ($r = 0.6$, $P = 0.001$; Fig. 3) were found. However, linear regression analysis showed that both chick weight and waiting time did not affect other selected organs weight in newly hatched chicks. The average quality score of all chicks was 95.19 ± 0.91 which had no relationship with waiting time and chick weight. The embryonic growth rate of the chick is related to the size of its egg and more specifically the difference in genetics and water content of the egg (Mortola and Al Awam, 2010 (Mortola and Al Awam, 2010). In addition, the broiler body weight in the first 6 days is highly correlated with final body weight at 6 − 7 weeks which indicates that a good start is very important to good overall performance in commercial broilers (Nir, 1995).

The range of CORT levels was from 4.24 to 19 ng/ml which was positive correlated with the time that

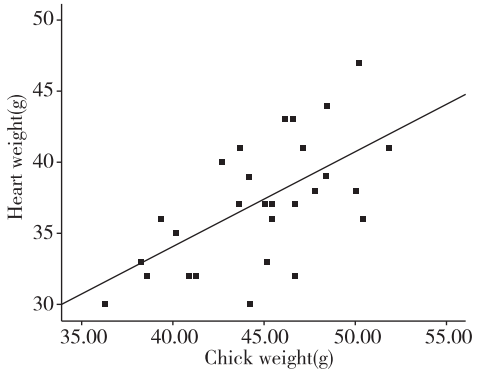

Fig. 2 Relationship between chick weight and heart weight in newly hatched broiler chicks (n = 29)

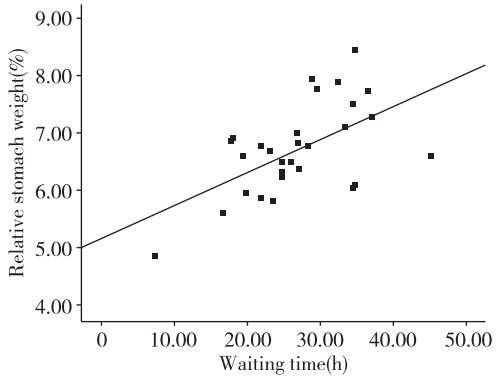

Fig. 3 Relationship between waiting time and relative stomach weight in newly hatched broiler chicks (n = 29)

chicks waited inside the incubator (r = 0.6, P = 0.03) (Fig. 4). Glucocorticoids (cortisol and corticosterone) are stress hormones and reflect the activity of adrenal glands. Plasma CORT levels of newly hatched chicks fluctuated between 6.5 to 11.5 ng/ml, but without differences within 12 hours post hatch (Scott et al., 1981). The highest levels of CORT of broiler chicks after being de-beaked and vaccinated at the hatchery are reported to be 20 ng/ml. In our study the chicks with more than 40 hours waiting time had higher CORT levels (up to 19 ng/ml) which were near the CORT levels of beak trimming chicks.

Conclusions

In our study the combination effects of egg weight and waiting time on chick weight were calculated and CORT levels relate to the waiting time. Chicks lost weight and had higher CORT levels when they stay longer in the incubators which could be influenced by stress or activity of those chicks after hatch. Therefore, it is very important to narrow the spread of hatch considering the animal welfare issues and chick quality.

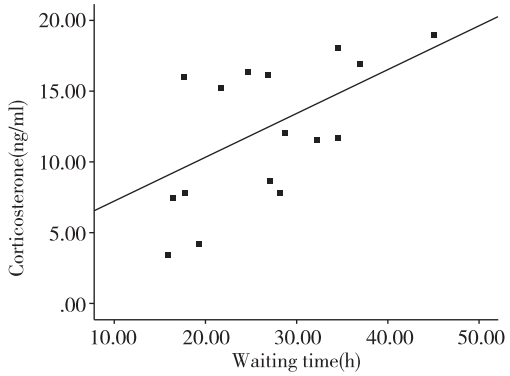

Fig. 4 Relationship between plasma corticosterone levels and waiting time in newly hatched broiler chicks (n = 15)

Acknowledgements

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siMMin™: On Line Software Tool to Simulate Zinc Balance in Feeding Programs of Growing Pigs

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Summary: Copper and zinc are essential nutrients that are usually supplied above nutritional requirements in pig diets. As a result, high Zn and Cu concentrations are found in animal wastes, which poses a risk of soil accumulation when pig manure is spread on arable land. Moreover, technological treatments of pig slurry concentrate zinc in the solid fraction and this by-product may exceed maximal authorised Zn content when used as organic fertiliser. The mass balance approach has been recently updated to measure the excretion of heavy metals (Cu, Zn) from pig production. siMMin™ has been developed with the support of INRA with the following objectives:

– the software should be intuitive and easily usable in any pig farm whatever the conditions;

– it focuses on the pig growing life, from the weaning until slaughter;

– calculation can be adapted to each user, taking into account farm variables of pig growth performance and feeding program;

– the software enables to simulate changes in each variable compared to the existing situation, in order to measure the rate of improvement in the total reduction of zinc in the life of the growing pig and the change in Zn concentration of animal waste;

– the calculator tool benchmarks any situation to existing EU regulation.

The software is on line since January 2013 at www. animine. eu/simmin/and can be utilised by all stakeholders involved in pig production.

Introduction

Copper and zinc are essential nutrients that usually have to be supplemented to pig diets so that animal requirements are fulfilled. Excess levels are supplied in practice either to secure sufficient safety margins, or because they can have beneficial effects at pharmacological dosage on gut health and growth performance. As a result, high Zn and Cu concentrations are found in animal wastes [1]. Zinc concentrations in pig manures have increased in China: from an average of 137 mg/kg dry weight in the 1990's up to 843 mg/kg in 2003 [2]. It poses a risk of metal accumulation in the soils when pig manure is spread on arable land. It is estimated that manure from livestock production contributes to 51% on the total Zn input on agricultural soils in China. Moreover, technological treatments of pig slurry concentrate zinc in the solid fraction and this by-product may exceed maximal authorised Zn value when used as organic fertiliser [3]. Some scientific methods to measure zinc balance in pig farms have been proposed by INRA to regulatory authorities and can be more widely communicated to the international pig industry.

Material and methods

In 2003 the first national references for the excretion of nutrients (N, P, K) and heavy metals (Cu, Zn) from pig production were agreed by French scientific and regulatory authorities [4]. Zinc body retention was calculated as:

$$Zn\ (mg) = 21.8 \times Body\ Weight\ (kg)$$

The effects of age, gender and pig breed on mineral accretion have been assessed [5]. When the minerals are expressed on a per kilogram of body component basis, Zn composition of loin and ham is similar for both genetic lines and sexes [6]. Dietary manipulation can affect Zn concentration in some organs [7] but should not markedly affect total carcass content. Zn bioavailability measured by accumulation in storage tissues can be affected by mineral source [8]. However, zinc source does not modify Zn balance [9,10] and Zn concentration in the pig carcass [11]. More recently, the mass balance approach has been updated from latest literature data [12]:

$$Zn_{body} = 20.6 \times Empty\ Body\ Weight$$

$$EBW = 0.96 \times Body\ Weight$$

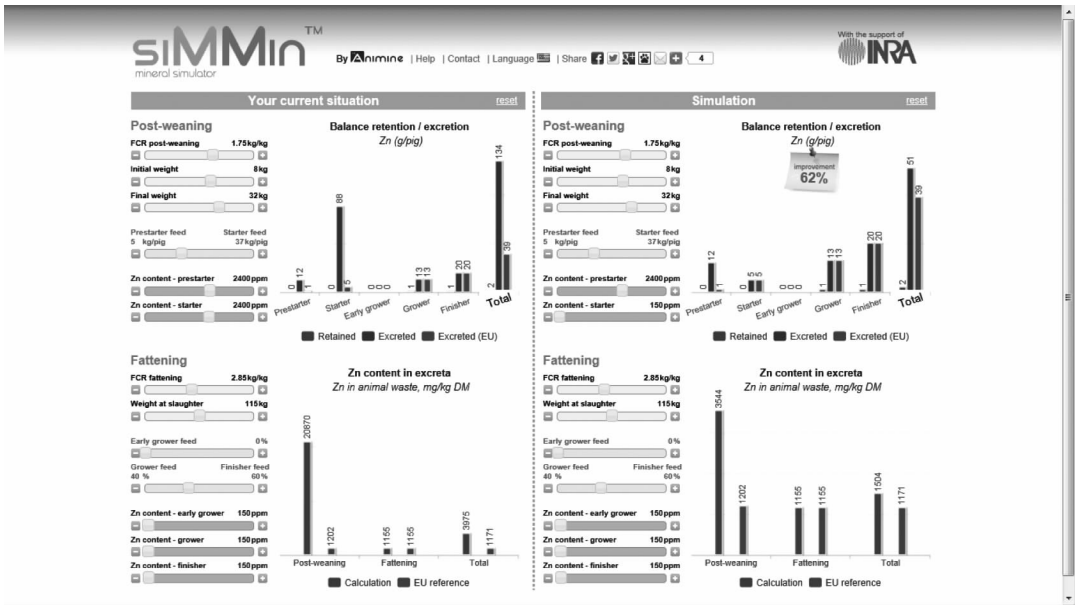
Thus, homeostatic regulation of Zn metabolism minimizes the effect of animal and diet in the assessment of the environmental balance in standard pig husbandry practices. As zinc retention in growing pig can be calculated based on the difference in mineral body content between the beginning and the end of a defined period, mineral excretion can be deduced by the difference between Zn intake and Zn retention.

siMMin™ has been developed with the support of INRA with the objective that it should be easily utilised

whatever the local conditions. For example, the feeding program in the fattening phase can be simplified to one single diet or phased with three diets (early grower, grower and finisher feeds). The same is possible for the post-weaning phase. It should be intuitive and user-friendly so that nutritionists and pig producers can get access and understand it. Users can insert their own data including animal performance (FCR, feed consumptions, initial and final body weights), and zinc dietary concentrations in each feed. The software enables to simulate changes in each variable compared to the existing situation, and to measure the rate of improvement in the total reduction of zinc in the life of the growing pig. siMMin™ also calculates the Zn concentration in pig waste. This calculator tool benchmarks any situation to existing EU regulation.

Results and discussion

siMMin™ appears on one single web page, which makes it user friendly:



Mineral balance can be assessed by the analysis of excreta [13] but the sampling of manure is labour intensive and results may not be representative [14]. Therefore, siMMin™ facilitates decision making without any need for animal experiments. As many pig producers do not know the trace mineral concentration in pig diets, this software favours the need for mentioning Zn contents on labels of feed bags in countries where this is not compulsory by local legislation. siMMin™ focuses on the pig growing life, from the weaning until slaughter; in order to be adapted to farrowing-fattening farms where significant volumes of manure also come from breeding pigs, it should also include results from sows.

The first version of siMMin™ is in English language and, depending on the level of interest expressed locally, should be later available in national languages for major pig producing countries.

Conclusions

The high supplementation dosage of zinc in pig diets may result in excessive levels in soils and waters in areas of intensive animal production. With siMMin™, all stakeholders in the pig production chain now have a user friendly tool to mitigate the environmental footprint towards more sustainable practices.

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Can Aggressive Behaviour among Piglets be Stopped by a Sound Signal and Reward Treatment?

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Summary: The aim of the study was to test whether aggressive actions among piglets could be redirected by an automatically generated sound signal followed by a sweet food reward. Per round, four litters of 25 days old suckling piglets (BHZP breed) were trained five times per day during eight days to expect a sweet food reward from a dog feeder after hearing a specific sound. In total 72 piglets in 12 entire litters were trained in 3 rearing rounds. After the training period, the piglets were weaned and mixed in two pens, 12 piglets per pen. Immediately after mixing and 24 h later the animals were visually observed by two trained persons (one per pen) disguised behind a transparent blind for 3 h. They released the sound signal and feeder when aggressive behaviour started. Video records were taken by 2 cameras, in total 36 h of video (6 h per pen, 3 rounds) for later analysis. During these 36 hours, 612 aggressive attacks were identified of which 55.3% could be redirected by the sound signal and reward treatment. The type of aggression and the moment in time during the aggressive action as well as the role of the piglet during a fight (initiator or receiver) influence significantly the effectiveness of the sound award system ($P < 0.001$). Initiator piglets stopped aggressive action more often after feeder activation (Odds Ratio = 0.75) than receivers (1.33). Attacks by jumping on the opponent could be easier redirected (0.55) than attacks with bites (0.61) and head trusts (0.67). Very few piglets responded to the sound signal (9.31) when the fight had already fully started. The chance to interrupt aggression by the sound signal and reward system decreased by 28% with every second passed from the start. The investigated sound reward system can reduce aggression in piglets when properly applied in time.

Introduction

As agonistic behaviour among unfamiliar pigs is necessary to develop a dominant hierarchy within a group, a minimal level of aggression in group-housed pigs at mixing cannot be completely eliminated. In many cases however, elevated forms of aggression have been reported by farmers and other professionals as one of the biggest problems of modern pig husbandry, mostly associated with the standard practice of repeated mixing of unfamiliar pigs. Although much of this aggression might be viewed as a harmless trial of strength (Huntingford and Turner, 1987) there can nonetheless be serious consequences including impaired growth, stress, wounds, poor meat quality and reduced animal welfare. Other serious animal welfare problems in intensive farming of fattening pigs are tail and ear biting. These abnormal behaviours can reduce production results, increase on farm costs (e. g. labour and treatment costs) and lead to a variety of physical damage and carcass condemnation resulting in financial losses for the farmer and the abattoir (Zonderland, 2010). Therefore there is a need to find a solution to control or limit these behaviours among pigs. In recent years, cognitive abilities of animals were widely tested (e. g. Held et al., 2005; Wredle et al., 2006; Jansen et al., 2008), showing that pigs can learn successfully to cope with difficult experimental tasks. The term of cognitive enrichment was used by Puppe et al. (2007) for an instrumental food-rewarding learning device, previously described by Ernst et al. (2005). With this device the pigs were trained to approach a feeder in order to receive a food reward whenever they were called at several, unpredictable times each day. These experiments showed that sound and feed are effective stimuli for the instrumental learning in pigs, and that the pigs can clearly and selectively successfully associate the sound and the feed reward. Thus, our idea was to use the associative instrumental learning based on classical conditioning techniques as an approach to reduce the incidence of aggressive and abnormal behaviours of pigs reared in intensive conditions. For this purpose we used a prototype of a food-rewarding device for cognitive enrichment, represented by an automatic dog feeder. The piglets learned to approach the feeder which released some attractive feed after hearing the sound signal. Main objective was to test the effectiveness of trained sound signals on redirecting pigs' attention from aggressive behaviour.

Material and methods

The aim of our study was to test the influence of cognitive enrichment during the execution of aggressive and abnormal behaviours after weaning and mixing.

The enrichment tool consisted of a commercially available electronic dog feeder (Manners Minder Treat and Train®) filled with potentially attractive feed for piglets (chocolate candies). The feeder was activated by remote control releasing a sound signal 2 s before feed distribution.

The study was conducted at the research farm Ruthe of the University of Veterinary Medicine Hannover, Foundation, Germany. Per experimental round, four litters of 25 days old suckling piglets (BHZP breed) with average weight of 7 kg ± 1 kg were trained five times per day during eight days to expect a sweet food reward from adog feeder after hearing a specific sound. In total 72 piglets in 12 entire litters were trained in 3 rearing rounds. The piglets had been raised from birth until weaning with their littermates and dam in a pen (1.80 m × 1.80 m) with partly slatted floor, equipped with farrowing crate, heated piglet area and provided with water and dry feed ad libitum. Two dog feeders were positioned on opposite walls of the selected pens on height of 0.8 m from the ground and contemporary activated by an observer from outside of the room. During the training period the dog feeders were activated every ten minutes. On the day of weaning from each trained litter 6 piglets were selected based on their weight (average 10 kg ± 1 kg) and mixed in two pens, 12 piglets per pen. The dimension of the pens was 2 m × 1.8 m with slatted floor and solid pen walls. The piglets had ad libitum access to dry food and water. Immediately after mixing and 24 h later the animals were visually observed by two trained persons (one per pen) disguised behind a transparent blind for 3 h. They released the sound signal and feeder when aggressive behaviour described in Table 1 started.

Table 1 Description of behaviours of the piglets leading to the activation of the feeder

Behaviour	Description
Fight	A fight lasts longer than a single aggressive interaction and begins with open-mouthed contact and ends when the pigs lose contact for at least 5 seconds (based on Erhard et al., 1997 and Gonyou et al, 1988). Series of mutual vigorous bites, pushes, head trusts occur from the pigs involved
Attack with Bite	Aggressive attack with biting any part of another pig without head trusting (after O'Connell and Beattie, 1999)
Push rooting disc	Pushing or ramming another pig with his rooting disc without biting, in an event that is not rated as part of a fight
Head trust	Ramming or pushing another pig with the head, with or without biting, in an event that is not rated as part of a fight (O'Connell and Beattie, 1999)
Chase	Pig is following another pig in quick pursuit, usually biting or trying to bite (Erhard et al., 1997), receiver pig withdraws or escapes
Lifting other	The pig puts its snout under the body of a pen mate (from behind or the side) and lifts the pig from the floor (after Morrison et al., 2003)
Jump on other	The pig starts an aggressive interaction jumping with his front feet on another pigs head-neck area (McGlone 1985)

Video records were taken by 2 cameras (Guppy F-080C and. Guppy GC1350 (Allied Vision Technologies, Germany), placed at the height of 2.0 m above the pens floor. In total 36 h (6 h per pen, 3 rounds) of recorded videos were analysed by one observer using the software "Labelling Tool" (Viazzi et al., 2011) developed in Matlab (R2009a, The MathWorks Inc., MA, USA). The following parameters were recorded: the exact duration of each behavioural event (the start and finish time); the behaviour of the piglets at the moment of feeder sound exposure (Table 1); the response of the pig on the feeder sound during the performance of the behaviour (0 = continued behaviour; 1 = interrupted behaviour, approached the feeder); the role of the piglets: initiator and receiver.

Data analysis included three rounds. Logistic regression was used to model the effect of duration as covariate on distraction. The estimated regression coefficient of the duration on distraction was used to estimate the predicted values of logits. A second model was fitted to evaluate the effect of behaviours on distraction. Due to the small number of events, push rooting disk and lifting other behaviours were excluded from analysis. The third model was used to evaluate the effect of the piglet's role (initiator; receiver) on distraction. Parameter estimates were obtained from the CATMOD procedure of SAS (2008). Odds ratios and predicted values of logits were calculated according to the methods of Hosmer and Lemeshow (1989). A risk is significant if the confidence interval does not include 1.0

Results and discussion

From the whole video database a total of 612 aggressive events were used for the analysis of which

55.3% could be redirected by the sound signal and reward treatment. The logistic regression showed that the type of aggression and the moment in time during the aggressive action as well as the role of the piglet (initiator or receiver) influence significantly the effectiveness of the sound award system ($P < 0.001$). The behaviours were included in the model as risk factors for the continuation of the behavioural event after the feeder sound exposure (Table 2). The results show the low risk of continuation of the specific aggressive behaviours such as, jump on other (Odds ratio (OR) = 0.56), attack with bite (0.61) or head trust (0.43) after the interruption. Regarding the behaviours of elevated aggression level, the risk of continuation raised twice in a case of chase (OR = 2.11); fight had a nine times risk of being continued after the feeder sound release (OR = 9.31).

Table 2 Response to the feeder sound: odds ratios for the aggressive behaviours

Behaviour	Estimate (β)	Odds ratio
Jump on other	−0.59	0.55
Attack with bite	−0.50	0.61
Head trust	−0.40	0.67
Chase	0.75	2.11
Fight	2.23	9.31

Initiator piglets stopped aggressive action more often after feeder activation (Odds Ratio = 0.75) than receivers (1.33). The logistic regression model predicted the continuation of behavioural event with a probability rate of 0.28 when the feeder sound was released within the first second after it's start (Fig. 1). The later the sound signal was released after the start of the behavioural event the higher is the predicted probability that the action or fight continues and the less is the probability of interrupting it.

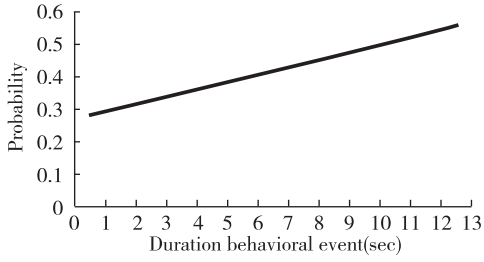

Fig. 1 Estimated probability that the behavioural event is continued the later the sound was released after its start The X axis is the duration of behavioural event (sec) at the moment of release of the sound signal. The Y axis is the predicted probability (P) of a continuation of the behavioural event after release of the sound.

A certain behaviour results when an effective stimulus is received or generated by the animal (Lehner, 1996). When one behaviour occurs, an ongoing behaviour may be inhibited, if both behaviours cannot be performed at the same time. It is obvious that for the inhibition of an ongoing behaviour the new stimulus should be stronger than the current one. In pigs, as in most other animals, food acquisition is highly motivating (McLean, 2001). The specific question in our study was if the sweet feed stimulus is strong enough to inhibit aggressive or abnormal behaviour and can redirect the animal to the feeder. The results show that highly aggressive behaviours such as chase and fight were less likely to be interrupted. The behaviours as jump on other, attack with bite and head trust were found to be successfully interrupted by the feeder sound when applied quickly. The explanation could be that the majority of the hierarchical fights occurred already on the first day, while during the second day short aggressive events dominated, which probably just were tests of strength (Huntingford and Turner, 1987) of dominant animals, which did not lead to any violent response of the receivers. The more time passed from the start of an aggression, the more the animals were involved in aggressive actions and the less was the probability to interrupt them by the sound signal.

Conclusion

In conclusion, the presented method bears some potential to reduce the frequency of aggressive actions among young piglets. An exception are aggressive behaviours related to the establishment of a dominance hierarchy within a group, they can rarely be interrupted as this study shows. Aggressive and violent actions among young piglets in unstructured common pens of livestock production systems, caused by reasons different from hierarchy establishment, can be successfully reduced by a sound food-reward application.

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Stress Caused by Transport in Farm Animals: Mechanisms and Consequences

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Summary: During transportation animals are exposed simultaneously to a variety of stressors which may reduce their welfare and have negative economic consequences. The term "stress" has been widely used in biology to describe a set of physiological and behavioural changes elicited by aversive stimuli. Both the hypothalamic-pituitary-adrenal (HPA) axis and the sympatho-adrenomedullary (SAM) system are generally considered to be the two main elements of the stress response. On the other hand, there is now sufficient evidence showing that it is not the physical nature of an aversive stimulus that has negative consequences on the animal but rather the degree to which the stimulus can be predicted and controlled. As a result, it has been suggested that the term stress should be restricted to conditions where an environmental demand exceeds the regulatory capacity of the organism, in particular when such conditions include unpredictability and uncontrollability. The stress caused by transport not only reduces the welfare of the animals but may also have negative economic consequences. These result among other causes- from transport-related diseases and tissue damage.

What is stress?

The term "stress" has been widely used in biology to describe a set of physiological and behavioural changes elicited by aversive stimuli. In 1929, Cannon described stress as the sympatho-adrenomedullary (SAM) system's attempt to regulate homeostasis when threatened by a variety of aversive stimuli or stressors. Later on, Selye (1936) conducted some of his classic studies on the response of the hypothalamic-pituitary-adrenal (HPA) axis to noxious stimuli and suggested that the organism reacted in a non-specific manner to a wide variety of aversive stimuli, mainly with an increase in the HPA axis activity.

There is now sufficient evidence showing that it is not the physical nature of an aversive stimulus that has negative consequences on the animal but rather the degree to which the stimulus can be predicted and controlled (Weiss, 1970). As a result, it has been suggested that the term stress should be restricted to conditions where an environmental demand exceeds the regulatory capacity of the organism, in particular when such conditions include unpredictability and uncontrollability (Koolhaas et al., 2011).

Current research on stress biology has addressed the role of the brain. Several areas of the brain are involved in the organization of responses to aversive or threatening stimuli, and these areas interact extensively. Neurons in the hypothalamus, for example, are sensitive to internal physicochemical stimuli and to external physical and psychosocial stimuli. To a great extent the stress response is mediated by the corticotropin releasing factor (CRF) that is secreted mainly by the paraventricular nucleus of the hypothalamus (Dunn and Berridge, 1990).

Stressors can be conveniently divided into physical stressors, social stressors resulting from the interactions with individuals of the same species and stressors related to handling by humans. Stressors have additive effects. This means that when several stressors impinge upon the animal at the same time, the resulting stress response will be much higher than if the animal was exposed to one stressor only. Therefore, circumstances such as transport can be particularly difficult for the animals.

Stress can be measured using behavioural and physiological parameters. The most obvious indicators that an animal is having difficulty coping with handling or transport are changes in behaviour which show that some aspect of the situation is causing distress. The animal may stop moving forward, freeze, back off, run away or vocalize (Grandin, 1980). It is important to realize, however, that the extent of behavioural responses to stressful situations varies from one species to another and also between individuals of the same species. (Broom and Johnson, 1993; Geverink et al. 1998). Once journeys start, some species of farm animals explore the compartment in which they are placed and try to find a suitable place to lie down. However, when the journey involves many lateral movements or sudden braking or acceleration, the animals cannot lie down. Therefore, the proportion of animals that remain standing during

transport is a relevant measure of welfare in relation to the roughness of the journey. For example, Ruiz de la Torre et al. (2001) found that more lambs remain standing during a rough journey than during a smooth journey. Another important behavioural measure of stress when animals are transported is the amount of fighting between animals.

Plasma levels of glucocorticoids, catecholamines, prolactin and endorphins as well as heart rate are among the most frequently used physiological parameters to study the effects of transport (Broom and Johnson, 1993). Levels of neurotransmitters in the brain - although less frequently used - are also of interest (Broom and Johnson, 1993). Acute phase proteins are also useful to assess the extent of tissue damage caused by transport (Saco et al., 2003).

Consequences of transport stress

The stress caused by transport not only reduces the welfare of the animals but may also have negative consequences in terms of economic profit and public health. Such consequences result among other causes-from transport-related diseases and tissue damage.

The most important transport-related disease is Malign Hyperthermia or Porcine Stress Syndrome, which is a serious welfare problem in pigs (PSS). This involves a cascade of physiological changes that may cause death. Stress and forced physical exercise may lead to an increase in body temperature, cardiac arrest and death. Death rates are higher when conditions are hot and wet. For example, it has been found that death rate increased with the outside temperature from 0.07% for outside temperatures lower than 5℃ to 0.11% for temperatures higher than 15℃. Death rate also increases with the length of the journey from 0.084% for journeys shorter than 75 km to 0.12% for journeys longer than 150 km (Colleu and Chevillon, 1999).

Apart from the environmental conditions and the length of the journey, the genotype of the pigs also has an effect on the death rate and it is now well established that reduction of the frequency of the halothane gene in commercial pigs would lead to a major reduction in pre-slaughter death rate (Murray and Johnson 1998). The halothane gene (Hal) is now considered to be equivalent to the *ryr-1* gene, which encodes a muscle protein called ryanodine receptor or calcium release channel (Fujii et al. 1991). Pigs with an altered form of the protein perform a prolonged muscle contraction (forced physical exercise) that induces increase in body temperature (hyperthermia). For example, one study carried out in Spain by Fàbrega et al. (2002) found that the frequencies of pre-slaughter deaths within each genotype were estimated in 0.02%, 0.09% and 2.29% for NN, Nn and nn genotypes, respectively. According to these results, the removal of both nn and Nn genotypes would imply an eleven times reduction in the pre-slaughter mortality rate (from 0.22% to 0.02%)

Salmonellosis in pigs is an important public health problem. It has been shown that transport stress increases the excretion of *Salmonella* by carrier pigs transported to the slaughterhouse and this in turn may cause contamination of shipping equipment and holding areas, resulting in pre-slaughter transmission of *Salmonella* to non-infected pigs (Isaacson et al., 1999; Boughton et al., 2007). Although the mechanism of this stress-induced excretion is not known, there are some indications that the stress-induced activation of the SAM system may play a role.

Tissue damage during transport often results from fighting. Mixing of unacquainted animals leads to an increase in agonistic activity in several species, such as cattle and pigs (McBride et al. 1964), due to the animals establishing a new hierarchy in the group.

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Model-based Prediction of Nephropathia Epidemica Outbreaks Based on Climatological and Vegetation Data and Bank Voles Population Dynamics

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Abstract: Nephropathia epidemica (NE) is a human infection caused by Puumala virus (PUUV), which is naturally carried and shed by bank voles (Myodes glareolus).

Objective: The objective of this paper was to develop a method that allows model-based prediction 3 months ahead of the occurrence of NE epidemics.

Data and Methods: Two datasets were utilised to develop and test the models. These data sets are concerned with NE cases in Finland and Belgium. In this study we selected the most relevant inputs from all the available data for use in a dynamic linear regression (DLR) model.

The number of NE cases in Finland were predicted based on the time series data of average monthly air temperature (℃) and bank vole's trapping index using a DLR model. The bank voles' trapping index data were interpolated using a related dynamic harmonic regression model (DHR).

For the Belgium case, no time series of the bank voles' population dynamics were available. Several studies, however, have suggested that the population of bank voles is related to the variation of seed production of beech and oak trees in Northern Europe. The NE occurrence pattern in Belgium was, therefore, predicted by using remotely sensed phenology parameters of broad-leaved forests, together with the oak and beech seed categories and average monthly air temperature (℃).

Results: The time variation in NE outbreaks in Finland could be predicted three months ahead with a 34% mean relative prediction error (MRPE).

NE outbreaks in Belgium were predicted three months ahead with a 40% MRPE, based only on the climatological and vegetation data.

Conclusion: NE outbreaks can be predicted by using dynamic data-based models with time-varying parameters. Such a predictive modelling approach might be used as a step towards the development of new tools for the prevention of future NE outbreaks.

Food Hygiene

Study on Quality of Raw Milk in Smallholder Dairy Farms in Jimma Town, South-Western Ethiopia

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Summary: A cross-sectional study was carried in Jimma town, western Ethiopia to assess the milk quality in 47 dairy herds during the months of December 2009 to January 2010. Bulk milk samples were collected from all 47 dairy herds once in a week for four consecutive times. Somatic cell count, total bacterial count, coliform count and antibiotic residues tests were performed using standard laboratory procedure. Bulk milk somatic cell count (BTSCC) was measured by using Delaval Direct Cell Counter (DCC) while enumeration of total bacterial counts (TBC) and coliform counts (CC) was done by inoculation on Petrifilm plates; antibiotics residues in milk was also determined by using Copan Milk Test (CMT). The geomean SCC in bulk milk was 6.25×10^5 cells/mL and the mean total bacterial count and coliform counts was 9.62×10^5 and 2.26×10^5 CFU/mL, respectively. A positive antibiotic residue was recorded in 55.3% of the studied herds. Significantly higher correlation was observed between TBC, and CC ($r = 0.71$, $P < 0.05$) of the milk quality parameters. The study revealed that milk produced in the study area is of poor hygienic quality and with antibiotic residues and for this reason raw milk should be used at least after boiling in the study area and farmers should get awareness on the negative effect of antibiotics on public health.

Key words: milk quality, somatic cell count, total bacterial count, coliform count, antibiotic residues, Jimma, Ethiopia

Introduction

Milk is a complex biological fluid and a good growth medium for many microorganisms [1]. Origin of bacterial contamination of raw milk could be either air, dust, the milkier hands, equipment, feed, soil, faeces or grass. Normally, the milk from the cow contains some harmless bacteria, if not diseased. High-quality milk contains a low number of somatic cells and a low bacteria count, and is free of human pathogens and antibiotic residues [2]. Examination for the presence and number of specific micro-organisms, somatic cell count, and antibiotic residues in milk is an integral part of quality control or quality assurance plan [1]. This may be applied to a number of areas: raw materials, intermediate samples, finished products, or environmental/equipment sites[1].

In Ethiopia, the Quality and Standards Authority of Ethiopia (QSAE) has prepared the Ethiopian standard on unprocessed whole/raw cow milk with bacteriological quality requirements. However, it is only functional for imported milk and milk products. Yet, the regulation in terms of hygienic limits for total bacterial counts (TBC), coliforms count (CC), and somatic cell counts which are the major indicators of milk quality is not implemented on local basis. So far the milk quality control available is limited to an alcohol and a specific gravity test; which are only functional by milk processing plants and cooperatives to receive milk from farmers. Milk and milk products may also be contaminated with antibiotic residues [3]. Antibiotics residues test is unknown or unknowingly ignored by most dairy industry in the country. In an area like Jimma, there is no quality control or quality assurance for raw milk supplied to the consumers.

A limited study done on bacteriological quality of raw cows' milk in Ethiopia indicates that the majority of bulk milk samples exceeded the international standard limit for raw milk [4, 5]. However, information available on milk quality at herd level in Jimma is lacking. This study were to assess the microbial quality of raw cow milk in Jimma (Ethiopia) based on the somatic cell count (SCC), total bacterial counts (TBC), and coliform counts (CC) and to determine the prevalence of antimicrobial residues in bulk milk in Jimma (Ethiopia).

Material and methods

Description of the study area

Jimma is found in Oromia Regional State, located at 352 km south-west of the capital, Addis Ababa, at latitude and longitude of 7°40′N 36°50′E. The annual rainfall of the area ranges from 1400 to 1900 mm, and the temperature ranges from 6 ℃ to 31 ℃. The area is mainly known by its mixed coffee-crop-livestock production system. The dairy production system practiced in the area is an urban smallholder dairy production system. Farmers keep cross-bred cows with varying blood level (data was not available). These farms had low milk production, poor health condition, high prevalence of mastitis and poor quality feed supply. Majority of the farms sell milk to milk retailers although few farms sell in their own milk shops.

Data collection

Study herd

Forty-seven smallholder dairy farms in Jimma town, Ethiopia, were included in this study. Selection of the farms was based on farmers' willingness to cooperate in research. The majority of animals kept in door in tie stall. No separate milking pen for milking cows. In all farms, either owner or employee performed hand milking twice a day: early in the morning and late in the afternoon. Either plastic or aluminum bucket was most commonly used for milking. Milk was not cooled until delivery and transported either by cart, car or man power for the retailers.

Milk sample collection

Bulk milk samples were collected from the milking bucket at farm level four times throughout a 2 month period (December 2009 to January 2010) from the afternoon milking. Milk was thoroughly mixed before sampling from the milking bucket. Then, 25 mL of the milk was collected aseptically into screwed sterile sampling bottle, labeled and stored in ice packed box and transported to Jimma University, College Agriculture and Veterinary Medicine, Microbiology laboratory for analysis. All tests were done on the day of sample collection.

Sample preparation and culture for bacteriological assessment

In enumeration of total bacterial count (TBC) and coliforms count (CC) for each sample a serial dilutions were prepared (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) in physiological saline water. Then dilutions were made by withdrawing 1 mL bulk milk of each sample with sterile disposable pipette into sterile tube containing 9 mL of physiological saline water. Consequently, for enumeration of TBC and CC a 1 mL of each dilution was transferred on Petrifilm plate by using sterile pipette. Subsequently, a plastic spreader was gently pressed on the center of the plate to distribute the sample evenly on Petrifilm. The Petrifilm plates were then kept in an incubator at 37℃ for 24 – 48 hours.

Quantification of colonies

Following incubation, plates exhibiting 25 – 300 colonies were counted for TBC using colony counter. All red colonies counted regardless of their size or intensity. The average number of colonies in a particular dilution was multiplied by the dilution factor to obtain the TBC. The TBC was expressed as the number of organism of colony forming units per mL (CFU/mL) of samples according to the standard procedures recommended by the manufacturer. Similarly, CC was also determined following the same procedure as in TBC. After incubation, plates exhibiting 10 – 100 colonies were counted for CC using colony counter and the total number of colonies found was multiplied by the dilution factor.

Somatic cell count

Bulk milk somatic cell count was measured by using DeLaval Cell Counter (DCC). Milk samples of 100 μL was taken into a special cassette, containing small amounts of reagents when mixed with the milk reacts with the nuclei of somatic cells and measuring the optic density. This is recorded as an image, and that image is used to determine the number of somatic cells in the milk which visible in DCC within a minute. Then the number observed was recorded as the number of somatic cells for a particular sample.

Detection of antibiotic residues

Copan Milk Test (CMT) was used to determine antimicrobial residues in bulk milk. Each raw milk samples was shaken thoroughly and 100 μL of milk sample was added directly onto the surface of the agar of the test vial using a special No Drop Count Pipette. Then the test vials incubated at 64 ± 1 ℃ for 3 hours. After incubation, the test vials were cooled to room temperature as recommended by the manufacturer. Colours were compared to reference colours; yellow colours indicate the absence of antibiotic residues and purple colours indicate presence of antibiotic residues.

Data analysis

The data collected in the study was stored in the Microsoft Excel (MS excel) and descriptive statistics such as means, frequency distribution and percentages were used to summarize the data by using SPSS software version 16.0. Bulk milk SCC, CC, and TBC data obtained were transformed into decimal natural logarithms to normalize frequency distributions. Means were compared by the General Lineal Model of SPSS. Correlations between different parameters were evaluated by calculating the Spearman's rank correlation coefficients (rho).

Results

Descriptive analysis

Milk quality study

The mean TBC of the bulk samples was 962, 488 CFU/mL ranging between 11,175 and 3,000,000 CFU/mL (Table 1).

The mean CC of the bulk milk samples was 226,380 CFU/mL ranging between 200 and 1,000,000 CFU/mL whereas geomean SCC was 625, 832 cells/mL ranging between 135, 837 and 1, 755, 518 cells/mL among the different herds. Antibiotic residues were recorded in 55.3% of the dairy farms and 20.2% of the collected bulk milk samples (Table 2). As SCC increases the antibiotic residues recoded increases in this study (Fig. 1).

Table 1 Descriptive statistics of milk quality parameters of smallholder dairy farms in Jimma town (n = 47)

Variable	Number	Mean	Minimum	Maximam	Standard Deviation
TBC (CFU/mL)	47	9.62×10^5	1.12×10^4	3.0×10^6	7.77×10^5
CC (CFU/mL)	47	2.26×10^5	2.0×10^2	1.0×10^6	3.13×10^5
SCC (cells/mL)	47	6.25×10^5	1.36×10^5	1.75×10^6	4.04×10^5

Table 2 Frequency of distribution of TBC, CC, SCC and antibiotic residual tests of bulk milk samples in smallholder dairy farms in Jimma town

Parameters/Category	Number of measurement (N = 4 × 47 = 188)	Number of farms (n = 47)[1] (percent)
BMTBC in CFU/mL		
< 5000	23 (12.2)*	0(0)*
5000 – 10000	20 (10.6)	0(0)
10000 – 100000	47 (25.0)	10 (21.3)
> 100000	98 (52.1)	37(78.7)
BMCC in CFU/mL		
< 50	55 (29.3)	0(0)
50 – 100	1 (0.5)	0 (0)
> 100	133 (70.2)	47 (100)
BMSCC in cells/mL		
< 200000	29 (15.4)	5 (10.6)
200000 – 400000	39 (20.7)	15 (31.9)
400000 – 750000	42 (22.3)	14 (29.8)
> 750000	78 (41.5)	15 (27.7)
Antibiotic residual in bulk milk		
No	150 (79.8)	21 (44.7)
Yes	38 (20.2)	26(55.3)

[1] herd on mean value of the four times measurement.

* numbers in parenthesis are in percent.

Statistical analysis

Milk SCC was negatively correlated with both TBC and CC although the correlation was not statistically significant (Table 3). Total bacterial count was positively correlated with CC (r = 0.71, P < 0.05) (Table 3).

Table 3 Spreaman rank correlation coefficient (rho) between TBC, CC, and SCC

Parameters	LnTBC	LnCC	LnSCC
LnTBC	1	0.71929***	−0.08159
LnCC		1	−0.03089
LnSCC			1

*** statistically significant (P < 0.001).

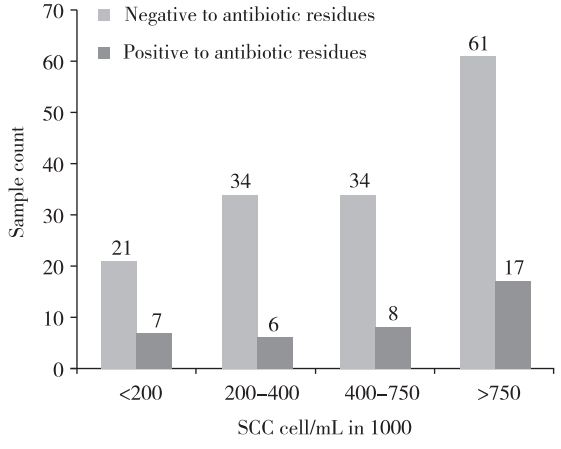

Fig. 1 Antibiotic residue status in bulk milk in different categories of SCC

Discussion

Basically, bacterial contamination of raw milk originates from two main sources; either from the cow or the environment [6]. Bacterial presence in milk have significant impact on the quality of dairy products and thereby with consumer acceptance. The mean TBC reported herein is 9 folds higher than the mean bacterial count observed in milk samples from milking bucket (1.1×10^5 CFU/mL) in central Ethiopia [4]. In this study, on 79% and 100% of the herds, both TBC and CC exceeded the acceptable limit for raw milk. This might be due to poor milking hygiene or use of dirty equipments and contaminated water before, during and after milking as it was also reported elsewhere [7, 8]. Previously, reported general hygiene at milking point has an effect on the numbers of micro-organisms in the milk [9]. Reports from Ethiopia also indicated 45.4% and 96% of the samples from bucket had TBC and CC greater than the international acceptable level of bacterial counts, respectively [5]. de Oliveira et al. [10] from Brazil reported high micro-organisms counts in the milk is an indication of poor sanitation and un hygienic conditions. Adverse effect in milk starts to become apparent when the total count approaches 10^6 and 10^7 CFU/mL. In the present study, 40.4% of the milk samples from bulk milk had higher than 10^6 CFU/mL TBC, almost similar with 44% reports from bucket milk in central Ethiopia, Debre Zeit [5].

About 42% of the herds included in the study had BMSCC within the current EU regulatory limit (<400,000 cell/mL) whereas more than 70% of the farms had BMSCC within the current US regulatory limit (<750,000 cell/mL). However, 28% of the studied herds SCC recorded in bulk milk were higher than the current US regulatory limit. Mastitis is characterized by an increased number of inflammatory cells, and SCC in milk is used as an indirect measure of the degree of the udder health [11]. This elevated BMSCC strongly indicates a high prevalence of intramammary infections in the herds in Jimma. Thus, further study is recommended to establish the Ethiopia SCC threshold for milk quality regulatory limit.

Antibiotic residues occur in milk supplies throughout the world [6]. In unregulated markets about 8% – 15% antibiotic residues exist in total bulk milk load [6]. In present study, antibiotic residues were detected in about half of the bulk milk samples. As SCC increases the antibiotic residues recoded increases in this study. Farms with higher SCC levels (>750,000 cell/mL) showed a much higher rate of antibiotic residue violations [12]. An increase in the incidence of mastitis in herd will generally result in increased use of antibiotics, which in turn increases the potential for antibiotic residues in milk. The high prevalence of antibiotic residues positive herds recorded in this study might be due to treated cows has been milked into the bulk milk before the withdrawal period is completed [6]. There is no published information on antibiotic residues in bulk milk in Ethiopia to the authors' knowledge and this is the first report in its kind. Therefore, further study is suggested to identify the extent of the problem in the study area.

The increased level of TBC in the bulk milk was accompanied by the increased CC. This is in agreement with the study done in USA [13]. The possible explanation for this correlation might be the potential contaminants of the bulk milk were the mastitis causing bacteria and unsanitary milking practices. Nevertheless, Pantoja et al. [13] reported a positive correlation between TBC and SCC, CC and SCC which has also negative correlation in this study but statistically insignificant. In another study in USA, paired correlation analyses between BMSCC and bacterial counts showed low correlations [14].

Conclusions

Milk produced in the study area is of poor hygienic quality and with antibiotic residues which requires special attention from public health point of view. Since this study was only focused on bulk milk, further work is recommended to elucidate some other factors that may have direct or in direct contribution to the poor hygienic quality of raw milk like knowledge of clean milk production, use and handling of milking equipment, and use of potable water for cleaning purposes in the study area. Based on the above conclusion the following points are recommended.

- Awareness creation to whole stakeholders in the importance of milk quality and steps required to achieve this goal.
- Awareness creation to farmers on the human health implication of the antibiotics in milk
- Technical support on milk test and formulation of quality control assurance with concerned bodies to curb the present problems

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Evaluation of Physicochemical Characteristics and Microbial Load of Raw Cow Milk in Arsi Zone, Ethiopia

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Summary: A cross-sectional study was conducted from July 2010 to May 2011 with the objective of evaluating physicochemical characteristics and microbial load of raw cow milk in Arsi zone, Ethiopia. A total of one hundred milk samples were collected from five milk collection centers and two dairy farms followed by laboratory analysis for physicochemical properties and microbial load. Variations in fat and total solids percentages among different collection centers were statistically significant ($P < 0.05$) with highest fat and total solids percentage recorded from Lemu Ariya primary dairy cooperative (4.83%) and Lemu Michael primary dairy cooperative (13.19%), respectively. Similarly, variations in total bacterial, coliform and somatic cell count showed statistical significant differences ($P < 0.05$) over the collection centers with the highest total bacterial (4.76×10^6 cfu/ml) and coliform (5.51×10^3 cfu/ml) counts recorded from Lemu Ariya primary dairy cooperative while the highest somatic cell count (9.93×10^3 cells/ml) are recorded from AMAE dairy farm. Variations in milk compositional and microbial quality observed across and within different collection centers and dairy farms calls for the need to gives focus on hygienic aspects of milk as improving production and productivity while planning interventions.
Key words: Arsi, cow, microbial, milk and physicochemical characteristics

Introduction

The safety of dairy products with respect to food borne diseases is a great concern around the world. This is especially true in developing countries where production of milk and various dairy products take place under rather unsanitary conditions and poor production practices [1]. In Ethiopia, in most cases, milk produced is marketed without any form of pasteurization or quality control measures and a considerable part of this milk is subjected to post harvest losses due to the high perishable nature of milk and mishandling [2]. There was no information on the physicochemical properties and microbial load of raw milk produced and marketed by dairy producers of Arsi zone. Therefore, this research work was conducted with the aim of providing baseline information on physicochemical properties and microbial load of raw milk in selected districts of Arsi zone.

Material and methods

Collection and analysis of raw milk samples

A cross-sectional study was carried out to collect one hundred milk samples from five primary dairy cooperatives (PDC) and two dairy farms located in three districts of Arsi Zone [3]. Physicochemical properties were determined by using Lactoscan 90 (Aple Industries services-La Roche Sur Foron, France) according to the manufacturer's instructions while the microbial count were determined following the standard procedures recommended by American Public Health Association [4].

Data management and analysis

Normalized microbial counts (after transforming to the log10) and physicochemical properties were analyzed by using General Linear Model (GLM) on SPSS version 15 statistical software and significant differences were considered as ($P < 0.05$).

Results and discussion

Fat and total solids variations across the milk collection centers were statistically significant ($P < 0.05$) (Table 1). Fat, lactose and total solids percentages were lower compared to the previous reports [5,6]. Variations in density, rate of adulteration and freezing point across the collection centers were statistically insignificant ($P > 0.05$). The raw milk freezing point in the present study agrees with the reports [6,7] while density of analyzed milk samples agrees with the previous reports [6]. Types of breed kept, differences in forage and feeding systems, milking frequency, milking method, seasonal changes and lactation period could be the possible reasons for the difference in physicochemical parameters of raw milk.

Table 1 Chemical composition of raw milk

Collection centers	N	Mean ± SD				
		Fat* (P = 0.004)	Protein (P = 0.104)	Lactose (P = 0.628)	SNF (P = 0.460)	Total solids* (P = 0.035)
Assela Town PDC	22	3.77 ± 0.69	3.13 ± 0.39	4.45 ± 0.67	8.28 ± 1.07	12.05 ± 1.64
Bilalo PDC	13	3.43 ± 1.09	3.24 ± 0.23	4.66 ± 0.42	8.60 ± 0.64	12.03 ± 1.16
Dosha PDC	13	4.13 ± 0.82	3.15 ± 0.11	4.51 ± 0.59	8.47 ± 0.50	12.60 ± 1.05
Dil-Betegbar dairy farm	13	3.71 ± 0.59	3.35 ± 0.04	4.83 ± 0.07	8.88 ± 0.12	12.60 ± 0.59
AMAE dairy farm	13	4.39 ± 0.93	3.32 ± 0.09	4.53 ± 0.90	8.55 ± 0.95	12.95 ± 1.04
Lemu Arya PDC	13	4.74 ± 1.48	3.23 ± 0.21	4.60 ± 0.38	8.53 ± 0.59	13.27 ± 1.28
Lemu Michael PDC	13	4.75 ± 0.71	3.27 ± 0.16	4.67 ± 0.28	8.64 ± 0.44	13.39 ± 0.85

* Fat and TSF percentage shows statistical significant differences ($P < 0.05$) among the collection centers.

Table 2 Total bacterial, coliform and somatic cell count of raw milk

Collection centers	N	Mean ± SD		
		* TBC (cfu/ml) (P = 0.000)	* CC (cfu/ml) (P = 0.000)	* SCC (cells/ml) (P = 0.009)
Assela Town PDC	22	$2.70 \times 10^5 \pm 5.31 \times 10^5$	$2.56 \times 10^2 \pm 3.60 \times 10^2$	$4.02 \times 10^5 \pm 5.45 \times 10^5$
Bilalo PDC	13	$2.09 \times 10^5 \pm 3.55 \times 10^5$	$1.64 \times 10^2 \pm 2.54 \times 10^2$	$1.80 \times 10^5 \pm 2.62 \times 10^5$
Dosha PDC	13	$2.03 \times 10^5 \pm 4.22 \times 10^5$	$1.71 \times 10^2 \pm 2.52 \times 10^2$	$4.28 \times 10^5 \pm 3.69 \times 10^5$
Dil-Betegbar dairy farm	13	$4.09 \times 10^5 \pm 4.92 \times 10^5$	$1.83 \times 10^2 \pm 2.47 \times 10^2$	$4.67 \times 10^5 \pm 5.79 \times 10^5$
AMAE dairy farm	13	$1.94 \times 10^6 \pm 3.61 \times 10^6$	$1.90 \times 10^3 \pm 3.61 \times 10^3$	$9.93 \times 10^5 \pm 9.00 \times 10^5$
Lemu Ariya PDC	13	$4.76 \times 10^6 \pm 5.05 \times 10^6$	$5.51 \times 10^3 \pm 5.05 \times 10^3$	$3.91 \times 10^5 \pm 4.35 \times 10^5$
Lemu Michael PDC	13	$3.31 \times 10^6 \pm 4.65 \times 10^6$	$2.05 \times 10^3 \pm 3.55 \times 10^3$	$2.91 \times 10^5 \pm 3.84 \times 10^5$

* TBC, CC and SCC shows statistical significant differences ($P < 0.05$) among the collection centers.

As indicated in table 2, the TBC were higher than the acceptable range between 2×10^5 and 4×10^5 cfu/ml set by American and European member states [4]. Factors that contribute to higher bacterial count could be poor animal health supervision, insufficient pre-milking udder preparation, insufficient cleaning of milkers' hands and milking utensils. Coliform counts were higher compared to the standards set by an American and European community member states [4]. This could be resulted from contamination of milk from manure, bedding materials, contaminated water, soil and inadequately cleaned milking utensil. Somatic cell counts were similar to the mastitis diagnostic threshold limit of Kenyan dairy herds [8]. As the concentration of somatic cells and microorganisms is too high in the milk of the teat cistern, forestripping is recommended prior to milking.

Conclusion

Variations in milk compositional and microbial quality observed across and within different collection centers and dairy farms calls for the need to gives focus on hygienic aspects of milk as improving production and productivity while planning interventions.

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Mycological Status of Egyptian Salted Fish

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Summary: Mycological qualities of Egyptian salted fish were evaluated. Twenty five samples of slated fish (*Hydrocynus forskalii*) were collected from Assiut city, Upper Egypt. Mean level of fungi obtained from muscular parts of examined fish was 1079.6 CFUs/g. A total of 30 genera, 75 species and some unidentified species of yeasts, dematiaceous hyphomycetes and sterile mycelia were isolated from all samples. *Aspergillus*, *Petromyces*, *Penicillium*, *Eurotium*, *Cladosporium* and yeasts were the most common fungi recovered on three media. Sensory quality, pH values and sodium chloride percentage were assessed.

Introduction

Salting is the most widespread and cheapest method for fish preservation. The salt curing is done by unscientific methods under unhygienic conditions. As a result, the products are grossly contaminated with dirt, sand, microbes and insect infestation and have only limited shelf life (Govindan, 1985, Ismail et al., 1994).

Fungal contamination of fish is considered the main cause of spoilage which leads to off flavour and unpalatable taste and may constitute a public health hazard as well as severe economic losses (Ward and Baaj, 1988; Dimond and Kendall, 2011).

Since salted fish constitute an important part of the diet of great portion of consumers in Egypt, therefore this study was performed to evaluate the mycological status of commercially available salted fish.

Material and methods

Collection of samples: Twenty five samples of salted fish (*Hydrocynus forskalii*, Cuvier 1819) were collected randomly from retail markets of different sanitation levels at Assiut city, Egypt, during the period from June 2010 to April 2011. The samples were transferred to the laboratory under aseptic condition with undue delay to be examined for their quality and fungal content.

Preparation of samples & estimation of sodium chloride percentage according to AOAC, (1980 and 1995).

Mycological examination: Fish samples were prepared according to the technique recommended by American Public Health Association (1985).

Three types of media were used for the isolation and enumeration of fungi: Dicloran rose-bengal chloramphenical agar medium (DRBC), Malt extract medium + 10% NaCl (MSA 10%) and malt extract medium + 20% NaCl (MSA 20%). Identification of mould genera and species were carried out on the basis of their macroscopic and microscopic characteristics following the identification keys of Domsch et al. (2007); Pitt and Hocking (2009).

Results and discussion

It is evident that all examined samples had NaCl content more than 6%. It was observed that the fungal propagates recovered on DRBC were higher than those recovered on malt extract agar amended with either 10% or 20% NaCl with the lowest count being recorded on 20% NaCl malt extract agar (Table 1 & 2). This may be attributed to the effect of high concentration of salt which inhibits the growth of many species of fungi. Similar findings recorded by Wheeler and Hocking (1993), Essa (1998) and Ahmed et al. (2005). Addition of sodium chloride at high rate to the medium gives the opportunity to halophilic moulds (which probably found in salted fish samples) to appear in such media.

This variation in mould counts in salted fish samples may be due to different levels of sanitary measures adopted during handling, manufacturing and storage. Addition of sodium chloride at high rate to the medium gives the opportunity to halophilic moulds (which probably found in salted fish samples) to appear in such media.

Conclusions

The incidence of moulds could be attributed to improper sanitation during catching, handling, processing, salting storage, transportation, distribution and marketing of fish. Contaminations with a variety of mould species resulted in undesirable changes of fish and rendering it unfit for marketing and increase the risk of infection with

Table 1 Minimum, maximum and mean (colony forming units) of fungal propagules recovered from commercially Egyptia salted fish on different media at 25℃

Media	Skin			Muscle		
	Min.	Max.	Mean	Min.	Max.	Mean
Dicloran rose bengal chloramphenicol	40	4020	929.2	0	14400	1079.6
10% NaCl malt extract agar	0	1140	152.08	0	282	73.92
20% NaCl malt extract agar	0	40	11.28	0	98	15.36

Table 2 Mean counts of fungal genera and species recovered from muscles of commercial salted fish

Taxa	Muscle											
	DRBC				MSA 10%				MSA 2%			
	TC	%TC	NCI	OR	TC	%TC	NCI	OR	TC	%TC	NCI	OR
Acremonium	220	0.82	7	L					2	0.56	1	R
Alternaria	520	1.93	10	M	12	0.62	4	L				
Ascotricha sp.	20	0.07	1	R								
Aspergillus	2260	8.39	19	H	324	16.77	14	H	36	10.06	8	M
Byssochjlamys spectabilis	200	0.74	3	L								
Cladosporium	1960	7.27	10	M	196	10.14	13	H	30	8.38	7	M
Cochliobolus lunatus	240	0.89	5	L								
Emericella	160	0.59	5	L	6	0.31	2	R	2	0.56	1	R
Eurotium sp.					368	19.05	13	H	170	47.49	11	M
Fusarium	720	2.67	8	M	10	0.52	4	L				
Graphium sp.	20	0.07	1	R								
Neosartorya fumigata		0.00			2	0.10	1	R	8	2.23	1	R
Nigrospora oryzae	40	0.15	2	R	4	0.21	2	R				
Penicillium	1000	3.71	16	H	112	5.80	7	L	96	26.82	4	L
Petromyces flavus	3500	12.99	17	H	676	34.99	12	M	10	2.79	2	R
Pseudoallescheria boydii	20	0.07	1	R								
Rhizopus		0.00			4	0.21	1	R				
Scopulariopsis	80	0.30	4	L								
Setosphaeria rostrata	40	0.15	2	R		0.00						
Sporothrix schenkii		0.00	1		2	0.10	1	R				
Stachbotrys	20	0.07	1	R								
Syncephalastrum racemosum	170	0.63	2	R	4	0.21	1	R				
Trichothecium roseum	40	0.15	3	R								
Sterile mycelia(dark & white)	100	0.37	11	L	26	1.35	2	R				
Yeasts	15620	57.96		M	186	9.63	5	L	4	1.12	2	R
Total	26950	100			1932	100			358	100		
No. of genera:	23	19				13				8		

TC = Total count; NCI = Number of cases of isolation; OR = Occurrence remarks; H = High; M = moderate; L = Low; R = Rare.

respective disease to consumers as a probable result of aflatoxins production by some fungal strains.

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Detection of Native and Modified Soybean in Some Meat Products in Assiut City, Egypt

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Summary: High meat prices prompted the meat industries in Egypt to produce various meat brands extended with soybean proteins. Genetically modified foods are often in the news. Agar gel immunodiffusion (AGID) and polymerase chain reaction (PCR) were used to detect soybeans in some meat products (minced meat, raw kofta, and sausage and beef burger). Soybean detected with AGID at 12%, 30% and 20% in raw kofta, sausage and beef burger, respectively, but not detected in minced meat. By using PCR native and modified soybeans detected at 100% and 69%, respectively in beef burger and at lower rates in other products.

Introduction

The use of soybean proteins as meat extenders in meat products has spread significantly due to the interesting nutritional, functional properties and economical reasons that are present in soybean proteins. There are many countries in which the addition of these proteins is allowed up to a certain extent.

Genetically modified foods are often in the news. Most of the developed analytical methods for GMO detection are DNA-based (Rodriguez-Lazaro et al., 2007)

Therefore, the objectives of this study were carried out for estimation of the adulteration in meat products in Assiut retail markets with soybean, detection of the accuracy of meat products labeling in the samples and comparison between the results of identification of species by AGID and PCR technique.

Material and methods

Samples

Two hundreds beef meat products samples of minced meat, raw kofta, sausages and beef burger (50 of each) were collected from Assiut city retail markets and analyzed for detection of meat adulteration with soybean.

Soybean antigens

Antigens from raw and heat treated soybean were prepared and kept frozen at −20 ℃ till be used.

Experimental animals

Female New Zealand white breed rabbits at (10 – 12 weeks old) were used. Rabbits were divided into 2 groups according to the number of antigens used. Three rabbits used for each group and 3 rabbits as control. Rabbits were immunized for production of the target antisera.

Preparation of soybean antigen & Immunization

As mentioned by Carp et al., (1999), Macedo-silva et al., (2000) and Zheng et al, (2007).

Methods for detection of soybeans

1-AGID Test: As mentioned by Siklenka et al, (2004).

2-PCR method: Fifty samples (14 samples of minced meat, 11 of raw kofta, 12 of sausages and 13 of beef burger) were chosen from the suspected and negative adulterated samples examined by AGID to be reexamined by PCR.

1. Extraction of DNA: By using QIAamp DNA Mini Kit (Catalog no. 51304, Qiagen Pvt. Ltd).

2. Polymerase chain reaction: Lectine gene used for amplication of sequence used for detection of native soybean and CP4EPSPS gene used for amplification of sequence used for detection of modified soybean (synthesized by Bio Basic inc.).

Species	Product size (bp)	Sequence	Name
		TGATGTGATATCTCCACTGACG	EPSPS-B1
Modified	172		
Soybean		TGTATCCCTTGAGCCATGTTGT	EPSPS-B2
		GCCCTCTACTCCACCCCCATCC	LE103
Native	118		
Soybean		GCCCATCTGCAAGCCTTTTTGTG	LE104

References for native and modified soybean is Lin et al, (2006)

LE Lectine gene

CP4EPSPS 5-enolpyruvylshikimate-3-phosphate synthase from *A. tumefaciens* strain CP4.

Results and discussions

Table 1 Incidence of native and modified soybean in the samples examined by AGID test

Type of soybean	Minced meat		Raw kofta		Sausage		Beef burger		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%
Heated soy	–	–	4	8	14	28	9	18	26	13
Raw soy	–	–	2	4	1	2	1	2	4	2
T. S.	–	–	6	12	15	30	10	20	30	15

Table 2 Incidence of native and modified soybean in the samples examined by PCR test

Type of soybean	Native Soybean		Modified Soybean			Total
	No.	N. S. %	No.	G. M. S. %	G. M. S. % from N. S.	
Minced meat	7	50	4	28. 6	57	14
Raw kofta	8	72. 7	5	45. 5	62. 5	11
Sausage	9	75	6	50	66. 7	12
Beef burger	13	100	9	69	69	13
Total	37	74	24	48	65	50

Out of fifty samples from each product suspected and negative adulterated meat products samples examined by AGID technique, an alternative method based on conventional PCR analysis to confirm the results of the adulteration which recorded by AGID technique. The results noticed that all samples give positive with genetically modified soybean were previously detected positive with the LE gene primer which present in all soybean products which also described by Lin et al. (2006).

Conclusions

It was cleared that native soybean or modified soybean can be easily detected by PCR in spite of the great processing of soybean before and after its addition. It was noticed that the sensitivity and accuracy of PCR in detection of soybean in meat products greatly overcome potency of AGID test.

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Spraying Efficacy of Slightly Acidic Electrolyzed Water for Reducing Microbial Contamination on the Surface of Eggs

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Summary: The efficacy of slightly acidic electrolyzed water (SAEW) to inactivate foodborne pathogens and indigenous microbiota on shell eggs were evaluated and compared to ClO_2 solution. Eggs were artificially inoculated with *S. enteritidis*, *E. coli* O157: H7 and *S. aureus* and sprayed with SAEW, alkaline electrolyzed water followed by SAEW (AKEW + SAEW), ClO_2 solution and deionized water for 10 s at room temperature. The effect of SAEW on the natural microbiota of eggs was also studied. SAEW, AKEW + SAEW, and ClO_2 solution treatments significantly reduced the population of *S. enteritidis* by 1.60 to 2.49, 1.88 to 2.52 and 1.52 to 2.07 $\log_{10}$CFU/g, *E. coli* O157: H7 by 2.21 to 2.78, 2.30 to 3.10 and 1.64 to 1.89 $\log_{10}$CFU/g, and *S. aureus* by 1.20 to 1.39, 1.25 to 1.41, and 1.09 to 1.35 $\log_{10}$CFU/g, respectively relative to control. The natural microbiota were reduced by 1.39 to 1.90, 1.51 to 1.97 and 0.74 to 1.33 $\log_{10}$ CFU/g for total aerobic bacteria, 1.50 to 1.79, 1.62 to 2.22 and 1.01 to 1.41 $\log_{10}$ CFU/g for coliforms, 0.99 to 1.86, 1.90 to 2.23 and 0.65 to 0.95 $\log_{10}$ CFU/g for Staphylococcus and 0.34 to 0.45, 0.38 to 1.22 and 0.04 to 0.31 $\log_{10}$CFU/g for moulds and yeasts, respectively. SAEW and ClO_2 solution with available chlorine of 60 mg/L showed no significant bactericidal difference for foodborne pathogens ($P > 0.05$), whereas the bactericidal activity of SAEW for *S. enteritidis*, *E. coli* O157: H7, total aerobic bacteria and Staphylococcus was significantly higher than ClO_2 solution at 80 mg/L and 100 mg/L of available chlorine ($P < 0.05$). SAEW was more effective in combination with AKEW. Results indicate that the disinfectant efficacy of SAEW is equivalent to or higher than that of ClO_2 solution and therefore SAEW shows the potential to be used for sanitization of egg shells as an environmentally friendly disinfection agent.

Introduction

There has been increased concern about the safety of foods especially products consumed fresh or slightly cooked. *Salmonella* spp., *Escherichia coli* O157: H7 and *Staphylococcus aureus* were reported to be the common foodborne pathogens that can cause human illness and death. Egg and egg product contamination, particularly by *S. enteritidis*, has been associated with many outbreaks of salmonellosis [1,2]. Therefore, an effective method for reducing or eliminating pathogens is crucial for food safety and human health. Clorinated water of 50 – 200 ppm is the most commonly used sanitizer in food industry [3]. However, chlorine is inactivated by organic material and can lead to the formation of potentially carcinogenic and teratogenic trihalomethanes and haloacetic acids [4].

Slightly acidic electrolyzed water (SAEW) is a novel disinfectant with a pH value of 5.0 – 6.5, which is generated by electrolysis of a dilute hydrochloric acid and/or NaCl solution in a chamber without a membrane. SAEW has been proved as an effective antimicrobial agent for inactivating *E. coli*, *S. aureus* and *Salmonella* spp. in vitro [5]. With a near neutral pH, SAEW application seems promising as it minimizes human health and safety issues from Cl_2 off-gassing, reduces corrosion of surfaces, and limits phototoxic side effects [6]. However, little is known about the efficacy of SAEW to inactivate microorganisms on shell eggs. The objective of this research was to evaluate the efficiency of SAEW to inactivate foodborne pathogens and indigenous microbiota on shell eggs and to compare the efficiency of SAEW and ClO_2 solution.

Material and methods

Bacterial cultures

Freeze-dried pure cultures of *S. enteritidis* (chicken feces isolate), *E. coli* O157: H7 (human feces isolate) and *S. aureus* (raw milk isolate) were obtained from the China Veterinary Culture Collection (CVCC, Beijing, China). The population of *S. enteritidis*, *E. coli* O157: H7 and *S. aureus* in each culture was approximately 9.0 $\log_{10}$ CFU/mL.

Preparation and inoculation of shell eggs

Eggs weighing 55 ± 2 g were collected from a 50-wk-old commercial layer chickens, and washed with tap water and a commercially available sanitizer for 1 min. For inoculation, eggs were individually dipped into the inoculum prepared by placing 0.1 ml of approximately 10^9 CFU/ml *S. enteritidis*, *E. coli* O157: H7 and *S. aureus* suspension into 200 ml of sterile 0.1% peptone water for 10 min, and allowed to dry under a laminar flow safety hood for 1 h at a room temperature.

Preparation of treatment solutions

SAEW was produced by electrolysis of 10% NaCl

(w/v) in the SAEW generator set at a current of 8, 10, 12A, respectively. Solution of chlorine dioxide (ClO_2) was also prepared by diluting in sterile deionized water.

Treatment of shell eggs with disinfectants

Incubated and unincubated eggs were placed in a sterile plastic egg flat and sprayed with each solution for 10 s (approximately 15 mL of solution per egg) using the hand-operated manual spray bottle and allowed to dry for 10 min.

Microbiological analysis

After the treatment, eggs were aseptically transferred into a sterile plastic bag containing 25 mL of sterile neutralizing buffer solution and gently rubbed by hand for 1 min. The egg was then removed, and the neutralizing buffer solution was serially diluted in sterile 0.1% peptone water. The viable bacterial populations of pathogens and indigenous microbiota were enumerated by plating 0.1 mL of the appropriate dilutions in triplicate on plates and incubating at 37℃ for 24 h before counting. The weight of the shell was measured to determine the colony-forming unit of per gram of eggshell + membrane (CFU/g) by the method reported by Bialka [7].

Statistical analysis

All data were subjected to the general linear model procedure of SPSS 17.0 software (SPSS Inc, Chicago, IL). Duncan's multiple range test was used to separate means using a level of significance of $P < 0.05$.

Results and discussion

Effect of SAEW on pathogen bacteria artificially inoculated on shell eggs

The initial populations on the surface of shell eggs were 7.01, 7.14 and 7.04 $\log_{10}$CFU/g for *S. enteritidis*, *E. coli* O157:H7 and *S. aureus*, respectively. Fig. 1 showed that spraying with DW, AKEW, SAEW, AKEW + SAEW and ClO_2 solutions at different concentration for 10 s resulted in a reduction in populations of *S. enteritidis* by 1.03, 1.25, 1.60 – 2.49, 1.88 – 2.52 and 1.52 – 2.07 $\log_{10}$ CFU/g, *E. coli* O157:H7 by 1.05, 1.43, 2.21 – 2.78, 2.30 – 3.10, and 1.64 – 1.89 $\log_{10}$ CFU/g, and *S. aureus* by 1.00, 1.13, 1.20 – 1.39, 1.25 – 1.41 and 1.09 – 1.35 $\log_{10}$ CFU/g, respectively.

All treatment groups had a significant reduction in *S. enteritidis*, *E. coli* O157:H7 and *S. aureus* when compared with the control group ($P < 0.05$), while no significant differences were found between DW and AKEW treatment ($P > 0.05$). As compared to DW, SAEW80, SAEW100, AKEW + SAEW and $ClO_2$100 can reduce the population of *S. enteritidis* significantly ($P < 0.05$), however, no significant differences were observed among SAEW80, SAEW100, AKEW + SAEW80, AKEW + SAEW100 and $ClO_2$100, nor were there any significant differences among SAEW60, AKEW + SAEW60, $ClO_2$60 and $ClO_2$80 ($P > 0.05$) (Fig. 1(a)). In comparison with DW, SAEW and AKEW + SAEW decreased the microbial population of *E. coli* O157:H7 significantly ($P < 0.05$), however there were no significant differences between DW and ClO_2 solutions ($P > 0.05$) (Fig. 1(b)). A significant reduction in the population of *S. aureus* was observed when comparing SAEW100 and AKEW + SAEW100 to DW ($P < 0.05$), while no significant differences were found between DW and ClO_2 solutions ($P > 0.05$) (Fig. 1(c)).

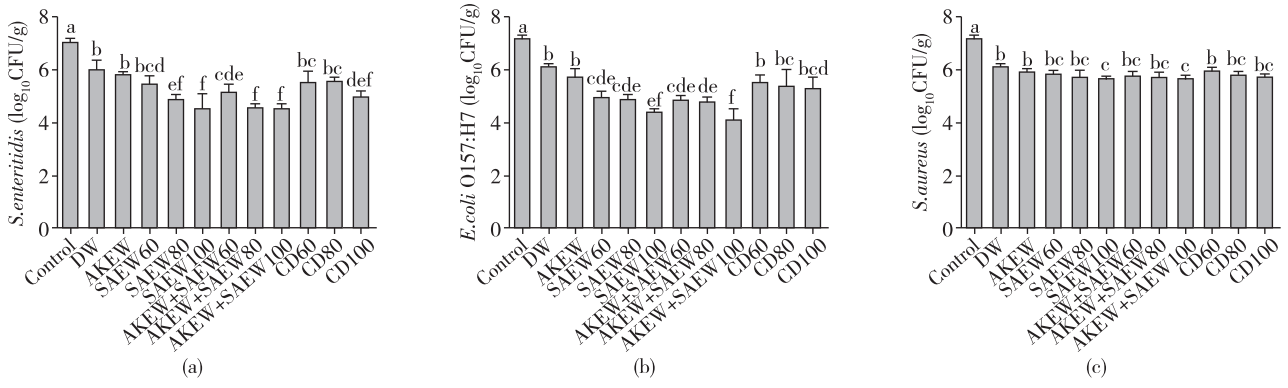

Fig. 1 Survival population of *S. enteritidis* (a), *E. coli* O157:H7 (b) and *S. aureus* (c) of shell eggs sprayed with different solution. The treatment solutions were deionized water (DW), alkaline electrolyzed water (AKEW), slightly acidic electrolyzed water (SAEW60, SAEW80, SAEW100), AKEW followed by SAEW (AKEW + SAEW60, AKEW + SAEW80, AKEW + SAEW100), solution of chlorine dioxide (CD60, CD80, CD100) at concentrations of 60, 80, 100 mg/l of ACC. [a-f]Means with different letters are significantly different at $P < 0.05$.

Effect of SAEW for reducing natural microflora on shell eggs

The microbial counts naturally present on shell eggs sprayed with DW, AKEW, SAEW, AKEW + SAEW and ClO_2 solutions for 10 s are shown in Table 1. The initial microbial populations of total aerobic bacteria, coliforms, staphylococcus and moulds and yeasts in control were 5.66, 5.37, 5.61, 4.37 $\log_{10}$ CFU/g, respectively. Compared with the populations of total aerobic bacteria in control, DW, AKEW, SAEW, AKEW + SAEW and ClO_2 solutions at the different available chlorine concentration decreased 0.5, 1.17, 1.39 – 1.90, 1.51 – 1.97, 0.74 – 1.33 $\log_{10}$ CFU/g, respectively. AKEW, SAEW, AKEW + SAEW, $ClO_2$80 and $ClO_2$100 reduced the populations of total aerobic bacteria significantly when compared with DW ($P < 0.05$). There were significant difference between AKEW + SAEW80 and ClO_2 solutions, whereas there were no significant difference between SAEW60, AKEW + SAEW60, $ClO_2$80 and $ClO_2$100 ($P > 0.05$). The population of coliforms on shell eggs was reduced by 0.26, 0.91, 1.50 – 1.79, 1.62 – 2.22, and 1.01 – 1.41 $\log_{10}$CFU/g for treatment of DW, AKEW, SAEW, AKEW + SAEW and ClO_2 solutions when compared with the control, respectively. In comparison with DW, SAEW, AKEW + SAEW, ClO_2 solutions reduced the population of coliforms significantly ($P < 0.05$). There were no significant difference between SAEW and ClO_2 solutions at the same available chlorine concentration ($P > 0.05$).

Table 1 Effect of different solution on the inactivation of total aerobic bacteria, coliforms, staphylococcus, and moulds and yeasts on shell eggs at different available chlorine concentration

Treatments	Surviving population ($\log_{10}$CFU/g)			
	Total aerobic bacteria	Coliforms	Staphylococcus	Moulds and yeasts
Control	5.66 ± 0.34^{a}	5.37 ± 0.42^{a}	5.61 ± 0.26^{a}	4.37 ± 0.31^{a}
DW	5.16 ± 0.31^{ab}	5.11 ± 0.12^{ab}	4.99 ± 0.21^{b}	4.27 ± 0.29^{a}
AKEW	4.49 ± 0.58^{cd}	4.46 ± 0.38^{bc}	4.67 ± 0.58^{bc}	4.14 ± 0.38^{a}
SAEW60	4.27 ± 0.16^{def}	3.87 ± 0.27^{cde}	4.62 ± 0.07^{bc}	4.03 ± 0.45^{a}
SAEW80	3.86 ± 0.30^{efgh}	3.74 ± 0.36^{def}	4.25 ± 0.35^{cd}	3.99 ± 0.27^{a}
SAEW100	3.76 ± 0.26^{fgh}	3.58 ± 0.26^{ef}	3.75 ± 0.06^{de}	3.92 ± 0.27^{a}
AKEW + SAEW60	4.15 ± 0.27^{defg}	3.75 ± 0.37^{def}	3.71 ± 0.18^{de}	3.99 ± 0.15^{a}
AKEW + SAEW80	3.57 ± 0.06^{h}	3.62 ± 0.25^{ef}	3.58 ± 0.04^{e}	3.2 ± 0.29^{b}
AKEW + SAEW100	3.69 ± 0.31^{gh}	3.15 ± 0.65^{f}	3.38 ± 0.75^{e}	3.15 ± 0.52^{b}
$ClO_2$60	4.92 ± 0.39^{bc}	4.36 ± 0.71^{cd}	4.96 ± 0.28^{b}	4.33 ± 0.26^{a}
$ClO_2$80	4.12 ± 0.22^{defg}	3.77 ± 0.33^{def}	4.83 ± 0.25^{bc}	3.86 ± 0.30^{a}
$ClO_2$100	4.33 ± 0.30^{de}	3.96 ± 0.05^{cde}	4.66 ± 0.38^{bc}	4.06 ± 0.26^{a}

Control (not treated), DW (deionized water), AKEW (akaline electrolyzed water), SAEW (slightly acidic electrolyzed water), AKEW + SAEW (AKEW followed by SAEW), ClO_2 (Chlorine dioxide solution) at concentrations of 60, 80, 100 mg/l of ACC respectively. Values reported as the means of triplicate measurements standard deviation. $^{a-h}$ Means in the same column with different lowercase letters were significantly different ($P < 0.05$).

Similar results were also obtained in the disinfection efficacy to shell eggs on the population of Staphylococcus. When compared to untreated control, DW, SAEW, AKEW + SAEW, and ClO_2 solutions reduced the population of Staphylococcus by 0.62, 0.94, 0.99 – 1.86, 1.90 – 2.23, 0.65 – 0.95 $\log_{10}$ CFU/g, respectively. SAEW80, SAEW100, AKEW + SAEW reduce the population of Staphylococcus significantly ($P < 0.05$), compared to the control, while no significant differences were found between DW and ClO_2 solutions ($P > 0.05$). It was found that AKEW + SAEW80 and AKEW + SAEW100 treatment significantly reduced the populations of moulds and yeasts ($P < 0.05$) relative to un-treated control group. No significant difference were found among DW, AKEW, SAEW, AKEW + SAEW60 and ClO_2 solutions ($P > 0.05$).

The results obtained in this work showed that SAEW with an ACC of 60 – 100 mg/L had the comparative or higher powerful bactericidal activity to reduce foodborne pathogens and indigenous microbiota present on shell eggs compared to ClO_2 solutions at the same available chlorine concentration. Cao et al [8] demonstrated that SAEW had similar bactericidal activities with AEW and NaClO solution at the same ACC and contact time. Zhang et al [9] implied that SAEW is an effective method to reduce foodborne pathogens on seeds and sprouts with less effects on the viability of seeds. Pangloli et al [10] suggested that SAEW could minimize cross-contamination and reduce the risk of *E. coli* O157 : H7 present on the produce. Issa-Zacharia et al [5] found that SAEW and NaClO solution had no significant sanitization difference against food pathogens on fresh vegetables. These results indicate that SAEW with a near neutral pH may be a potential sanitizer represent an alternative to ClO_2 and

NaClO solution used in food industry.

Conclusions

The study demonstrates that SAEW has an equivalent or higher efficiency to reduce *S. enteritidis*, *E. coli*, *S. aureus* and indigenous microbiota present on shell eggs compared to ClO_2 solutions at the same available chlorine concentration. Therefore, SAEW shows the potential to be used for sanitization of egg shells as an environmentally friendly disinfection agent.

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Isolation Incidence of Several Non-cholera *Vibrio* Species from Aquatic Mollusks Harvested in the Black Sea Shore Area

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Summary: During this study, it has been investigated the frequency of isolation for non-cholera *Vibrio* spp. from aquatic mollusks. Samples have been collected from either the aquatic medium or the commercial network. This study continues the work of a previous study, with the aim to provide enough statistical data.

Between 2004 and 2011, a number of 931 samples were collected and prepared for analysis, consisting of mollusks obtained from the aquatic medium. Another batch consisting of 398 samples was obtained from the commercial network.

From the total number of 1329 samples collected an analyzed, 36 strains of non-cholera *Vibrio* spp. were isolated, representing a frequency of 3. 17%. The annual frequency of isolation was: 3. 42% for 2004; 2. 91% for 2005; 3. 31% for 2006; 3. 23% for 2007; 2. 34% for 2008; 3. 03% for 2009; 4. 02% for 2010 and 2. 96% for 2011.

The isolation frequency of *Vibrio* spp. for the Black Sea shore area was 1. 47%.

Introduction

The bacterial species pertaining to *Vibrio* spp. with a clinical importance (pathogens for the human being as well as for different species of animals) are usually present in the environment, forming a local micro flora in area with lakes, swamps, estuaries, seas and oceans, in the area with temperate and tropical climate [3, 14]. Scientists consider that these species are ubiquitous, being able to survive and multiply in the most unusual and different media. In the food products, the main cases when these species were isolated involved fish, mollusks and crustaceans samples [3, 10, 11].

Lately different proof has been subjected to attention, consisting of different reservoirs of *Vibrio* spp., in natural environments (sediments, plankton). These bacteria are further on taken and concentrated in the organs of the aquatic animals. The studies undertaken in the seashores of the U. S. A. and Australia revealed the existence of certain non-cholera *Vibrio* spp. reservoirs in crustaceans and shelled mollusks. This is the reason why in several countries such as the U. S., the main source of infection for humans is represented by the oysters. Also, several episodes of foodborne illnesses appear as a consequence of marine food products, mainly unprocessed meat of different aquatic species [1].

During preparation, for example in a restaurant's kitchen where all hygiene rules are respected, the number of bacterial cells for *Vibrio parahemolyticus*, may increase with $2 - 3 \times \log_{10}$. Therefore, a person who consumes 100 g fresh mollusks may ingest with it a bacterial load of $10^5 - 10^6$ bacterial cells, reaching the minimum dose required for an infection with *V. parahaemolyticus* Kanagawa-positive [2,3].

The prevalence of foodborne illness episodes induced by non-cholera *Vibrio* spp. may reach in countries such as Japan, a value of over 70% from the total number of bacterial infections [1]. The number of cases for each episode may vary from one to 100 individuals. These studies formed the basis of our research, in order to acknowledge the existence of *Vibrio* spp. in the salted waters existent on the Romanian territory, as well as the frequency of isolation for the different types of natural environments and from biological samples [5 – 10, 12, 13].

Material and methods

Different samples of mollusks species were collected from the natural environment of the Black Sea shore line, or from the marketplaces existent in Constanta (frozen samples).

The samples were collected constantly on a period of 8 years, while each year the examination was undertaken on a representative number of samples (931 samples mollusks from the Black Sea shore line and 398 samples taken from the marketplaces).

The samples collected from the aquatic medium were represented mainly by Lamellibranchia (mussels and oysters) from the following species: *Mytilus galloprovincialis* (Lambert), *Mytilaster* sp., *Corbula mediterranea* (Costa), *Cardium edule lamarcki* (Reeve), *Tellina tenuis* (Costa), *Mya arenaria* L.,

Dreissena polymorpha.

Occasionally oyster species from the Black Sea shore line were collected such as: *Ostrea taurica* and *Ostrea sublamellosa*.

From the marketplaces, frozen sea products were collected, represented by muscle portion of cephalopods (*Sepia* sp. cuttlefish, *Loligo vulgaris* squids, *Octopus* sp.) or chilled lamellibranchia (mussels and oysters) such as *Ostrea edulis* and *Ostrea sublamellosa*.

The sample collection was performed as it follows: 109 fresh samples and 37 chilled/frozen samples in 2004; 138 fresh samples and 34 chilled/frozen samples in 2005; 94 fresh samples and 57 chilled/frozen samples in 2006; 110 fresh samples and 45 chilled/frozen samples in 2007; 111 fresh samples and 60 chilled/frozen samples in 2008; 112 fresh samples and 53 chilled/frozen samples in 2009; 119 fresh samples and 55 chilled/frozen samples in 2010; and 138 fresh samples and 57 chilled/frozen samples in 2011 (Table 1 and Table 2).

The samples were prepared through special methods, for the isolation and identification of the bacterial species pertaining to *Vibrio* spp., using in comparison different methods such as: STAS ISO 8914, FAO-WHO methods, Oliver synthetic methods, original methods [1, 4, 5, 6, 8].

Table 1 Sample collection from the aquatic medium in Sulina-Sf. Gheorghe-Black Sea shore line region

Area	Collected species	Number of samples/year							
		2004	2005	2006	2007	2008	2009	2010	2011
Sulina	*Mya arenaria* L.	3	9	7	8	9	7	8	9
	Dreissena polymorpha	9	8	11	7	10	9	7	8
	Tellina tenuis (Costa)	5	5	6	4	3	4	5	5
Sf. Gheorghe	*Mya arenaria* L.	10	7	9	9	6	10	7	7
	Dreissena polymorpha	6	11	11	10	7	9	7	11
	Tellina tenuis (Costa)	5	5	3	4	5	2	4	5
	Corbula mediterranea (Costa)	3	4	4	2	4	3	5	4
Cap Midia	*Mytilus galloprovincialis* (Lambert)	9	18	5	16	12	17	8	18
	Mytilaster sp.	4	5	2	1	4	3	1	5
	Corbula mediterranea (Costa)	6	3	4	5	4	6	2	3
	Cardium edule lamarcki (Reeve)	2	4	2	1	4	2	1	4
Năvodari	*Mya arenaria* L.	1	2	1	1	2	4	5	2
	Mytilus galloprovincialis (Lambert)	12	17	7	14	10	12	16	17
	Mytilaster sp.	6	7	4	5	7	3	8	7
	Corbula mediterranea (Costa)	3	2	3	4	1	4	2	2
	Cardium edule lamarcki (Reeve)	4	2	3	2	1	4	5	2
Mamaia	*Mytilus galloprovincialis* (Lambert)	8	11	4	9	10	5	8	11
	Mytilaster sp.	2	4	2	1	1	2	4	4
	Corbula mediterranea (Costa)	3	2	1	2	1	1	3	2
	Cardium edule lamarcki (Reeve)	2	3	1	1	2	1	3	3
	Ostrea sublamellosa	3	5	2	3	4	2	5	5
	Ostrea taurica	3	4	2	1	4	2	5	4
Total number of collected samples		109	138	94	110	111	112	119	138

Table 2 Samples collection from the marketplaces in Constanţa

Preservation methods	Collected species	Number of collected samples/year							
		2004	2005	2006	2007	2008	2009	2010	2011
Frozen samples	*Octopus vulgaris*	3	2	6	5	4	7	5	6
	Octopus sp.	5	4	5	6	9	8	5	5
	Sepia officinalis	4	5	7	5	8	7	9	7
	Loligo sp.	6	10	12	8	11	7	10	12
	Ostrea sp.	5	3	7	4	6	8	8	7
	Other species	5	4	8	5	7	3	5	8
	Total	28	28	45	33	45	40	42	45
Chilled samples	*Ostrea taurica*	3	2	4	2	5	6	5	4
	Ostrea sublamellosa	3	2	4	3	5	4	7	4
	Ostrea edulis	3	2	4	7	5	3	1	4
	Total	9	6	12	12	15	13	13	12
Total number of collected samples		37	34	57	45	60	53	55	57

Results and discussion

From a total number of 1329 samples collected in a period of over 8 years, 42 strains of *Vibrio* spp. were isolated, being observed an isolation prevalence of 3.17%.

The most frequently isolated species was *V. alginolyticus* (21 strains), followed by *V. parahemolyticus* (14 strains) and *V. vulnificus* (7 strains). The highest frequency of isolation was observed on samples collected from the aquatic medium (36 strains), in comparison to the ones collected from the marketplaces (6 strains).

The evaluation of the isolation frequency for the Vibrio spp., considering the total prevalence and the annual prevalence is included in table 3.

Table 3 Annual incidence of non-cholera *Vibrio* spp. from mollusks, in each area of sample collection

Sample collection area	Total number of collected samples	Total prevalence (%)	Annual prevalence (%)							
			2004	2005	2006	2007	2008	2009	2010	2011
Sulina	166	3.47	5.88	0	4.17	5.26	0	5.00	5.00	0
Sf. Gheorghe	199	2.91	4.17	0	3.70	4.00	4.55	0	4.35	0
Cap Midia	199	2.99	0	3.13	7.14	4.17	0	3.12	5.88	3.13
Návodari	196	4.17	4.0	7.14	0	4.00	0	8.69	3.23	7.14
Mamaia	171	6.34	9.52	0	8.33	5.88	9.09	7.69	7.14	0
Market places in Constanta	398	1.47	0	5.88	1.75	0	1.67	0	1.82	5.88
Southern area (total number of collected samples)	1.329	3.17	3.42	2.91	3.31	3.23	2.34	3.03	4.02	2.96

Conclusions

1. The total isolation prevalence of non-cholera *Vibrio* spp. for the entire period of analysis was 3.17%. The annual variations were limited to 2.34% and 4.02%.

2. Through the statistical analysis of the obtained data, a higher isolation frequency of non-cholera *Vibrio* spp. was observed in the seashore line area (with a maximum value of 6.34% in Mamaia), in comparison to the fluvial areas (a maximum of 3.47% in Sulina).

3. The isolation frequency of non-cholera *Vibrio* spp. from the stored mollusks collected from the marketplace in Constanţa was lower (1.47%), in comparison to the mollusks samples collected from the aquatic medium. The annual variation of the isolation frequency has wide ranges, this fact drawing attention to the potential of mollusks' contamination, especially those stored an placed on the market. Furthermore, the multiplication of the *Vibrio* spp. strains is highly possible, directly on the aquatic products.

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Detection of *E. coli* O157: H7 from Local Beef Meat Products and Poultry Meat Products in Duhok Province Markets using Conventional Culture and PCR

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Summary: The study included detection of *Escherichia coli* o157: H7 in local beef and poultry meats represented with minced meat, beef burger and kebab for beef while the whole chicken, chicken burger, drum stick, breast and wings represented poultry meat samples, during the period from November 2011 till June 2012. Detection of these organisms depends on culture characteristic of colonies and biochemical tests, as well as using of polymerase reaction assay (PCR) as recent technique for confirmation. The serotyping depends on the presence of the gens *rfbO*157 and *flicH*7. Out of the total 100 sample, *E. coli* was isolated *rfbO*157 gene in 1 sample (2%) of local poultry meat while beef samples was negative for this gene. Also we detected *flicH*7 gene in 2 samples (4%) in imported beef whereas poultry meat revealed this gene (2%). DNA concentration of *E. coli* strains isolated from beef and poultry meat was between 67. 7 ng/μl and 53. 8 ng/μl respectively. We attained specific duplicated band with molecular weight 625 bp for *flicH*7 gene while *rfbO*157 gene revealed 259 bp.

Introduction

Meat has traditionally been viewed as a vehicle for a significant proportion of human foodborne disease. Although the spectrum of meat – borne disease of public health importance has changed with changing production and processing systems, continuation of the problem has been well illustrated in recent years by human surveillance studies of specific meat-borne pathogens such as *Escherichia coli*, *Salmonella* spp., *Campylobacter* spp. and *Yersinia enterocolitica*. In addition to existing biological, chemical and physical hazards, new hazards are also appearing [18]. *Escherichia coli* O157: H7 is an important human pathogen causing haemorrhagic colitis, haemolyti c-uraemic syndrome and thrombotic thrombocytopenic purpura [10,19]. *E. coli* O157: H7 serotypes are identified as enterohaemorrhagic *E. coli* and categorized as verotoxin-producing *E. coli* [11]. Verotoxin is also known as shiga-like toxin [3]. Cattle, especially the young ones, have been implicated as a principal reservoir of *E. coli* O157: H7 [17,20,15]. Cattle frequently excrete these bacteria in their faeces [16,9]. The illness is often linked to the consumption of contaminated and undercooked beef meat. Shiga toxin-producing *E. coli* (STEC) is now a major cause of food-borne disease, mostly in the United States, Canada, Japan and Europe [5,10]. The objective of the present study was to isolate *E. coli* O157: H7 from our local Meat products samples by conventional culture method and confirm it by a serogroup-specific PCR assay in Duhok province.

Materials and methods

A hundred samples were collected from different markets in Duhok province, fifty samples of meat products are beef and fifty samples of poultry meat products, in the period between Nov. 2011 and March 2012.

Culture conditions

Meat samples (10 mg each) were enriched in 90 ml of modified Tryptic Soy broth (mTSB) both supplemented with novobiocin (Oxoid, UK) at 37℃ for 18 hours. The broth cultures were spread plated onto MacConkey agar (LABM, UK) 37℃ for 18 – 24 hours for isolation of Enterobacteriaceae. Then sub cultured on Eosin Methylene Blue agar(LABM, UK) as selective media for 18 – 24 hour at 37℃, the colonies appear in Green Metallic sheen color in presence of *Escherichia coli*, followed by sub culturing on Hemorrhagic colitis agar (HC) (Sifin, Germany)[21].

PCR assay

The PCR assay has been carried out in Duhok research center in, Faculty of Vet. Medicine, Duhok university. NSF colonies on HC agar that had been confirmed as *E. coli* employed as templates for PCR assay. A whole-cell suspension was prepared by suspending a NSF bacterial colony from HC agar in sterile distilled water. The Bacterial DNA was extracted using Genomic DNA purification kit (Fernentas, Germany). 2 μl of the supernatant was used as template for amplification by PCR. The presence or absence of *flic*H7 gene encoding the flagella antigen H7 and *rfb*O157 gene

encoding the somatic antigen O157 [2, 12] were examined.

Table 1 Describes oligonucleotide sequence of primers used in the PCR reaction mixture

Target gene	Primer sequence (name)	Product size (bp)	Thermocycling programme
*rfb*O157	F: 5′-CGG ACA TCC ATG TGA TAT GG-3′ R: 5′-TTG CCT ATG TAC AGC TAA TCC-3′	259	94℃ for 5 min;94℃ for 60 s, 56℃ for 30 s, 72℃ for 60 s, 35 cycles; 72℃ for 10 min[7]
*flic*H7	F: 5′-GCG CTG TCG AGT TCT ATC GAG-3′ R: 5′-CAA CGG TGA CTT TAT CGC CAT TCC-3′	625	

The PCR reaction was performed in a 25 μl amplification mixture consisting of 12.5 μl master mix (Cinnagen), 8.5 μl of water and 1 μl (10 pmol) of each primer and 2 μl of template DNA. The thermocycler program was started with an initial incubation at 94℃ for five min, followed by 35 cycles of denaturation at 94℃ for 60 sec, annealing at 56℃ for 30 sec and elongation at 72℃ for 60 sec, and a final extension at 72℃ for 10 min. The PCR products were separated by electrophoresis on 1% agarose gel at 100 V for 40 min in TBE buffer, visualized by ethidium bromide staining, illuminated by UV-transilluminator and documented by a gel documentation apparatus. One-hundred bp DNA ladder was used as a size reference for PCR assay.

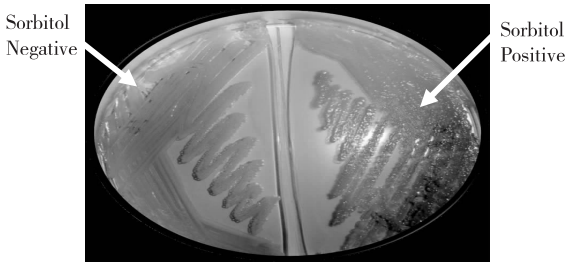
Result and discussion

The overall percentage of the Bacterial isolation had reached 1% of *Escherichia coli* O157: H7.

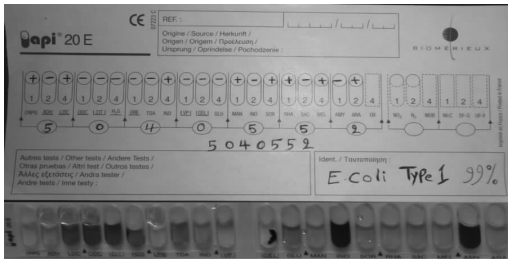
Table 2 Bacterial isolation rate from different source as shown down

Type of product	No.	Incidence %		
		Conventional	PCR	
			rfbO157	*flicH7*
Beef meat products	50	40% (20)	0% (0)	4% (2)
poultry meat products	50	16% (8)	2% (1)	2% (1)
Total incidence	100	28% (28)	1% (1)	3% (3)

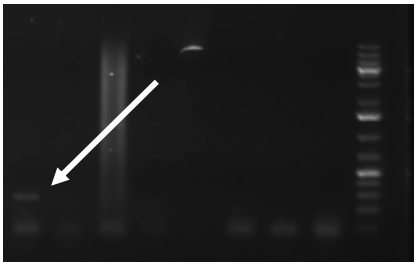
The overall percentage of bacterial isolation of *Escherichia coli* O157: H7 was 3%. Although *Escherichia coli* is part of the normal large-bowel flora of humans and animals. Most strains of *E. coli* are non-pathogenic in the intestine, some can produce diarrhea and by a number of distinct mechanisms. *E. coli* is usually considered to be an opportunistic pathogen which constitutes a large portion of the normal intestinal flora of humans. This organism can, however, contaminate, colonize, and subsequently cause infection of extra-intestinal sites and is a major cause of septicemia, peritonitis, abscesses, meningitis, and urinary tract infections in humans [1]. Although consider opportunistic pathogens, these organisms produce significant virulence factor such as endotoxins that can mediate final fatal infections. However, because they generally do not initiate disease in health, uncompromised human hosts, they are considering opportunistic. Additionally, in the case of Enterohaemorrhagic *E. coli* (EHEC), life threatening system disease can result from infection. Furthermore as the leading cause of nosocomial infections among enterobacteriaceae *E. coli* is likely to have greater virulence capabilities than the other species categorized as "opportunistic" Enterobacteriaceae [4]. In an earlier study, STEC O157 was isolated from 3.7% of retail beef and 1.5% of pork samples in the United States and Canada [3]. Although most sporadic cases and outbreaks have been reported from developed countries, human infections associated with STEC strains have also been described in Latin American countries, including Argentina, Chile and Brazil [10, 6]. It has also been reported from Kenya, Turkey and Iraq [13, 14]. We found that 28% of the samples were contaminated with the suspected *E. coli* O157 : H7 using conventional method and only 3% in using PCR, as it sensitive technique and more accurate. Our results suggested that cattle could be a reservoir of *E. coli* O157: H7 in Iraq, like many other countries and the products are contaminated during processing. [17, 20, 15]. Flagellar and somatic antigens can be detected by immunological assays. The main advantage of the employed PCR method is its ability to detect rough isolates or the isolates having a masked O antigen [2]. It has been described that the HC agar (Fluorocult HC Merck) The agar plates are clear and purple. Sorbitol fermentation and the pH-indicator, bromocresol purple, are used to detect sorbitol positive colonies yellow in colour. and MUG is split by the b-D-glucuronidase positive bacteria blue fluorescence under UV light 365 nm.. On HC Agar *E. coli* O157 : H7 colonies are colorless and show no fluorescence [8].



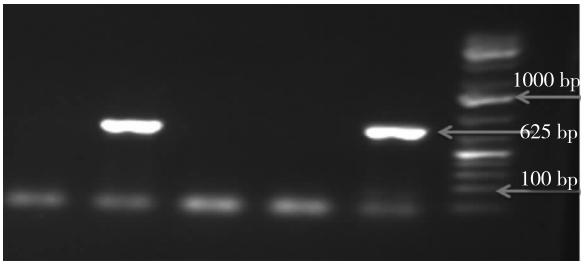
The picture shows the HC agar contain sorbitol negative *E. coli* and Sorbitol



The Picture above shows the positive result of *Escherichia coli* on API 20 E kit



Results of the PCR assay, amplifying 259 bp segment of rfbO157 of *E. coli* O157:H7. Lane 9 shows the sample.



Results of the PCR assay, amplifying 625 bp of flicH7 segment of *E. coli* O157:H7. Lane 1 and lane 2 shows the sample.

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Levels of Some Heavy Metals in Slaughtered Anmal's Tissues

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Summary: A total of 168 samples of liver, kidney and muscles were collected from cattle and buffaloes slaughtered in Assiut abattoirs. The samples were examined for the concentrations of heavy metals in foods by using the Atomic Absorption/Flaming Emission Spectrophotometer (Shimadzu model AA 630-02). Heavy metals levels higher than Egyptian monitoring standards were determined.

Introduction

In recent years, much attention has been focused on the concentrations of heavy metals in foods in order to check for those hazardous to human health. The industrial and edible species have been widely investigated were analyzed for Cadmium, Copper and Zinc (Kreuzer et al., 1988; Youssef et al., 1988; Lopez Alonso et al., 2000; Mansour and Sidky, 2002; Farkas et al., 2003). The aims of the present study were to determine the levels of Cadmium, Copper and Zinc in muscle, liver and kidney of cattle and buffalo slaughtered in Assiut city.

Material and methods

A total of 168 samples of liver (part of caudate lobe), kidney and muscles (part of diaphragm) were collected from 23 male cattle and 33 male buffaloes (2 – 3 years old) slaughtered in Assiut abattoirs. Each sample was about 50 grams weight and was individually placed in polyethylene bags and labeled with the date, kind, age and sex of each animal. The collected samples were immediately taken to the laboratory in an ice box where they were kept deeply frozen at –20℃ until preparation, digestion and analyses by using the Atomic Absorption/ Flaming Emission Spectrophotometer (Shimadzu model AA 630-02), using an air acetylene flame and hallow cathode lamp A. O. A. C. (1975).

Results and discussion

The obtained results indicated that there is a very highly significant difference in cadmium, concentration in cattle muscle versus buffalo muscle.

Values were always clearly less in meat than organs. There is a very highly significant difference in cadmium concentration between cattle liver versus cattle muscle. Besides, there is a highly significant difference in cadmium concentration between cattle kidney versus cattle muscle. No significant difference in cadmium concentrations between liver and kidney. Also, organs of buffaloes had no significant differences in their cadmium content.

Cadmium, Copper and Zinc levels are higher than monitoring standards obtained by Egyptian standards (2008) in organs and muscles of were detected in 18% in buffalo & cattle (Table 1).

Table 1 Percentages of samples that contain heavy metals higher than that recommended by the Egyptian Standards*

Heavy metal	Buffaloes			Cattle		
	Liver	Kidney	Muscle	Liver	Kidney	Muscle
Cadmium	18	1	10	13	13	0
Copper	25	3	3	26	1	0
Zinc	3	15	18	12	15	28

* Permissible limit (Egyptian Standards, 2008): Cadmium = 0. 1 mg/ kg, Copper & Zinc = 15 mg/kg.

Conclusions

Monitoring programme must be carried out periodically to determine the changing risks to health from animal food products in Assiut Province in order to inform any adopted management strategy. There are two practical options for reducing the levels of exposure to the local production of heavy metals from contaminated meat. The first is to take action against the emission sources themselves. The second option is to attempt to reduce human intake of contaminated meat.

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Proposals for Improvement the Traceability Concept

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Summary: This paper has as main objective the improvement and completion of traceability concept.

To support the new elements proposed, all papers found on Internet on this subject were studied, the diagram flows of the feed and feedingstuffs, animal holdings, food and foodstuffs establishments, also distribution and commercial units from Romania were studied, and integrated as inputs.

We need feed to obtain animals, these are reared and breed, slaughtered, some of these are intended for human and animal consumption, the others processed and the third are used for industrial purposes, all being traded. We propose to define these areas as domains of traceability: the feed, animal, food, industrial, commercial and finally consumer domains of traceability. In each domain there are different steps: eg: for animal domain-newborn, suckling weaned, young, growing or fattening. We propose to define these stages as chains of traceability. Also, we propose to define the connected activities from each chain of traceability as stage of traceability, the pursuit of all food products from feed chain to consumer chain as flow of traceability and for a single product as line of traceability. When food products are pursued from a given stage to its origin, we are talking about ascendant traceability and from this point to the final consumers, descendent traceability. We propose to define the itinerary of animal products along the flow of traceability as administrative traceability, the information and documents accompanying these products as informative traceability and to define the transport activities among subsequent chains of traceability as knots of traceability. When we follow only one product along the traceability flow, we are talking about channel of traceability, but when all products are involved, it means global traceability. The pursuit of the products inside a chain of traceability defines internal traceability, versus external traceability, outside of it.

Key words: traceability, chain, stage, knots

Introduction

Traceability concept is, together with HACCP concept, the cornerstone of food safety.

The traceability concept was defined by several authors in the field of food safety, as well as, by certain national and international institutions as ANSESS, OIE, EFSA, FAO, ISO and Codex Alimentarius. The most applicable definition is done by ISO 22000: 2005-Food safety management systems, ISO 9000: 2005-Quality management systems-Fundamentals and vocabulary and by Codex Alimentarius-CAC/GL 60-2006-Principles for traceability/Product tracing as a tool within a food inspection and certification system, European Commission-Council Regulations 852/2004/CE, 853/2004/CE, 854/2004/CE, 882/2004/CE.

Despite very conclusive definitions and developments to establish the structure of traceability concept, taking into account the new practical aspects in the food safety sector, the concept can be improved defining new elements of its structure, which is the purpose of this paper.

Material and methods

Colloquial defined as "from stable to table" or "de la fourche à la fourchette" the traceability concept has been structured within certain details.

We need feed to obtain animals, these are reared and breed, slaughtered, some of these are intended for human and animal consumption, the others processed and the third are used for industrial purposes, all being traded. We propose to define these areas as domains of traceability: the feed domain, the animal rearing domain, food domain, industrial domain, commercial domain and finally consumer domain of traceability. It is, in some extent, different from definition given to *food chain* done by ISO 22005: 2007 which involves in food chain only food, industrial, commercial and consumer domains. We consider our proposal for traceability more appropriate with reality.

Bolnot F. H. and Fleurynck Catherine defined *administrative traceability* as being the stages and processes through a product passes from its logical origin to the final consumer. It means that administrative traceability contains all domains of traceability proposed by us. It is quite different from commercial traceability,

pointed by ISO 22005:2007, point 3.6.

We propose to define all traceability domains for a product, meaning stages and processes through it pass from its logical origin to the final consumer as traceability elements. It is, in some extent, different from flowchart defined by ISO 22000:2005, points 3.4 and 3.8 which are referring to a certain domain of traceability and not to full traceability defined by us.

In the way of its traceability, a product is accompanied by documents (health certificate), information (advertising, origin and producer), labelling and means of its identification (ear tag).

We propose to define them as tools of traceability or logistic traceability which is different from term of *traceability system* defined by ISO 22005:2007, point 3.12 which referring only to information about a certain product, and it is in line with statements specified at point 3.6 of the same ISO, even if Bolnot F. H. and Fleurynck Catherine were spoken about *documentary traceability* or *informational traceability*.

Bolnot F. H. and Fleurynck Catherine defined *ascendant or "amonte" traceability* as being the following of a product, as administrative and tools of traceability, from a certain point to its logical origin and *descendent or "aval" traceability*, meaning the same thing but from certain point to its final consumer. The point/domain/stage (see below) from where the traceability of a product starts, either as ascendant traceability to its logical origin or descendent traceability to final consumer is defined by as origin of traceability. It is quite different from logical origin but same times can be similar.

At the same time they defined *global traceability* as being the traceability of more products having the same origin (meat, skin, organs and milk, derived from a slaughtered or live cow), *total traceability* as being the traceability of all products having the same origin.

We understand the logical origin of a product starting with feed domain, it means when it is talking about milk, the logical origin of milk is feed used by a cow to produce milk and final consumer, a human being or animal eating the milk or the place when a product is used (skins or leather for clothes).

We propose to nominate a new element of traceability called traceability branch/line, as being the way passed by a certain product along the all domains/chains of traceability from its logical origin to final consumer. It means, for instance milk, its traceability branch consists in feed domain (feed for feeding, minerals, feed supplements, etc.), animal rearing domain (drugs, vaccines), food domain (for raw milk)/industrial domain (for processed milk), distribution and trade domain, to the final consumer (human being, animals, cosmetic industry, etc.).

The branches traceability for several products having the same origin is similar to *global traceability* definition given by Bolnot F. H. and Fleurynck Catherine and the branches traceability for all products having the same origin is similar with *total traceability* defined by the same authors.

Inside a domain/chain of traceability there are specific activities or processes to with a product is submitted (eg. for food domain of pork there are pre-slaughtered examination, stunning, bleeding, evisceration, organs and carcases examination, skinning or scalding, drying, carcass identification,, boning, packing, storing and freezing). We propose to define these all activities and processes as stage of traceability. When several activities or processes, among all stages of traceability inside a traceability domain are involved we defined this activities and processes as traceability segment.

The clear definition of a stage of traceability and a segment of traceability, among the other stages of traceability within a domain of traceability is very important, for certain product traceability and there is a direct relation between a stage of traceability and critical control points and control point defined for HACCP.

When it is talking about the traceability inside a domain of traceability, along all stages of traceability of that domain, we defined this sort of traceability as internal traceability, against external traceability meaning the traceability of this product outside concerned domain of traceability.

In order to have a cow, for instance, we need animal genetic materials provided from animal domain, as well as, feed for feeding, supplied by feed domain, other materials for feeding and health or dietary supplements, purchased from chemical or pharmaceutical domain.

When the traceability of these all products, concurring to obtain and rear a cow, is followed, it means and we define this approach as conjunctive traceability, because several products having different origins compete to obtain a single product-a cow.

When an animal is slaughtered, several products derived from the process: hair, skin, claws, meat, fat, organs, bones, bowels, endocrine glands, brain, tongue, etc. are obtained and each of them passes through food domain (to be eaten as food or feed) or industrial domain (to be processed obtaining derived processed products used as food or feed or other domain of traceability-cosmetic industry, pharmaceutical industry, etc.). We defined the traceability branches of these derived products as disjunctive traceability, because a lot of products derived from a certain origin-a slaughtered and each of them has its specific branch of traceability to different final consumers.

Usually, between different domains of traceability or

even between stages or segments of traceability, there are transport activities. We propose to define the transport activities between domains of traceability as knots of traceability. These are very important because, during the transport activities, same negative influences on a product may happen with alteration its quality or safety.

When an animal is slaughtered several products derived from the process: hair, skin, claws, meat, fat, organs, bones, bowels, endocrine glands, brain, tongue, etc. are obtained, same of them are used for direct feeding or food or processed, emerging in distribution and trade domain, the others are processed for industrial, chemical or pharmaceutical purposes, emerging in industrial, chemical or pharmaceutical domains.

If, for instance, certain products are traced only for one domain, we propose to define this kind of traceability, individual/specific traceability (eg. milk, meat, fat, organs for food and feeding human beings or animals or bones and skins for chemical domain).

If all products derived from the same origin (eg. a slaughtered cow) are followed along their traceability branches inside all designated domain, it means that it is talking about common traceability.

Concerning the tools of traceability, they may be consisting in labelling system (eg. for feed and feedingstuffs, food and foodstuffs of animal origin or non animal origin), certification system conformity certification for feed and feedingstuffs or food and foodstuffs, health certification for live animals, animal origin and non-animal origin products, stamping system for animal origin products or for particular use products, identification and registration system for live animals, codification system for animal diseases and animal origin derived products.

Results and discussion

Food safety sector is one of the more sensitive components of word security strategy, creating, along the time, major crises with health, social, economical, financial and even political consequences. As a result, this domain is very well regulated and structured, in this process being involved a lot of international organisations and institutions, as well as, national specific bodies.

This paper is an attempt to improve and complete the concept of traceability, establishing detailed elements and tools, making it more practical and more feasible both for stakeholders and for technical and regulatory bodies.

Our proposals to improve the definitions and structures of traceability concept for food safety sector have been made taking into account the achievements obtained till now, avoiding parallelism and superposition with existing ones.

Our proposals are made combining the existed definition and structure of food safety traceability with practical observations within food industry.

Conclusions

1. We assessed almost all official information and regulatory items existing in relation with food safety traceability in order to be aware about the state of play in this field.

2. We've searched a lot of food safety industries, feed producing industry, animal husbandry, food processing sector, some industrial sectors using animal and non-animal food origin products to find specific aspects which are not specified in existing standards or guidelines concerning food traceability.

3. We tried to propose some definitions and elements of food traceability with the aim to complete the traceability concept for food and feed sector.

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Screening of Microflora in Rejected Broiler Chicken Organs

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Summary: The aim of this study was to link the assessment of pathogenic and conditional pathogenic microorganisms with different types of lesions discovered during the poultry slaughtering process.

Harvested 13 livers, 6 spleens, 21 kidneys and 10 hearts with gross lesions (steatosis, perihepatitis, multifocal military necrotic hepatitis, degenerative and vascular reactions and kidney conjunctive infiltration and heart hemorrhagic and degenerative lesions) from young slaughtered poultry were submitted to microbiological exam.

Selective culture media such as: CIN (Schiemann) agar, Wauters agar, MacConkey agar, Christensen media, Simons agar were used for pathogenic and conditional pathogenic microorganisms detection.

In case of the poultry livers *Pseudomonas* spp. (5/6), *Enterococcus* spp. (3/6) and yeasts (4/6) were correlated with military necrotic hepatitis; meanwhile non-specific hepatopathies were linked to *E. coli* (6/7), yeasts (5/7) and *Enterococcus* spp. (5/7).

The specific microflora for spleens was *Pseudomonas aeruginosa* (2/3) in correlation with hemorrhagic aspects and yeasts (2/3) with normal aspect, respectively. The detected microflora for kidneys was *E. coli* (8/21), yeasts (12/21) and *Enterococcus* spp. (2/21). For all samples of hearts no microflora was detected.

Yeasts were isolated from the majority of the samples; probably they have exogenous origin coming from the slaughtering process, manipulation and transport of samples.

Introduction

The organs of broiler chicken (liver, spleen, kidney, heart) are an important foodstuff due to the high amount of nutrients contained. The typology of organs' lesions correlated with the identification of microorganisms provides important information about health status of the flock and potential damage to consumers' health.

Offal consumption is not negligible in European Union. Data from the Comprehensive European Food Consumption Database; Concise Data Base summary statistics-Total Population, show that the consumption of edible offal, including poultry liver, heart, kidney and spleen, is between 1 g/day in Ireland and 26. 1 g/day in Poland, with an average of 7. 12 g/day for the European Union, considering the countries that participated to the survey [2].

The European Legislation impose a careful monitoring of all factors of the technological flow, which could in any way affect the finished product, by applying the principle from farm to fork. Meat inspection and in particular edible offal inspection is one step of that monitoring.

For food from animal origin, the main cause of exclusion from consumption because of a risk for public health is the contamination with microorganisms.

Material and methods

This research was conducted on rejected broiler chicken organs (liver, spleen, kidney, heart), using the same examination protocol for all types of samples.

Organs with gross lesions from young slaughtered poultry (13 livers, 6 spleens, 21 kidneys and 10 hearts) were collected from a large sized slaughterhouse from Dambovita county Romania.

First, gross assessment was performed for all samples.

All samples were submitted to histological exam (Masson trichromic and hematoxylin eosin stain) and microbiological exam (selective culture media CIN (Schiemann) agar, Wauters agar, MacConkey agar, Christensen media, Simons agar, XLD agar, gelose SS, Czapek media).

The isolated bacterial strains identification was performed on API or mini Vidas galleries system.

Results and discussion

Histologically, 7 livers showed non-specific hepatopathy, as steatosis (3/6) and haemorrhages (4/6). Six livers were associated with multifocal military necrotic hepatitis expressed as perihepatitis (4/6) and necrotic foci (2/6).

Nonspecific hepatopathy was featured by erythrocytes, normal or degenerated hepatocytes, many nude nuclei proving cell fragility. Multifocal miliary necrotic hepatitis expressed normal or degeneration of hepatocytes, inconstant occurrence of heterophils and mononucleated cells.

Microbiologically, *Pseudomonas* spp. (5/6), *Enterococcus* spp. (3/6) and yeasts (4/6) were correlated with military necrotic hepatitis; meanwhile non-specific hepatopathies were linked to *E. coli* (6/7), yeasts (5/7) and *Enterococcus* spp. (5/7).

Spleens showed normal aspect (3/6) and blood flow disturbances as haemorrhages (3/6).

The specific microflora for spleens was *Pseudomonas aeruginosa* (2/3) in correlation with haemorrhagic aspects and yeasts (2/3) with normal aspect, respectively.

All kidneys presented haemorrhages and conjunctive infiltration, the detected microflora for was *E. coli* (8/21), yeasts (12/21) and *Enterococcus* spp. (2/21).

Hearts were associated with haemorrhages and for all samples no microflora was detected.

All results were summarized in Table 1

Table 1 Correlation between contamination with microorganisms and lesions of organs

Microorganism/ Number of samples with lesions	LIVER		KYDNEY	SPLEEN		HEART
	Military necrotic hepatitis	Non-specific hepatopathies	Haemo-rrhages	Haemo-rrhages	Normal aspect	Haemorrhages
Enterococcus spp.	3/6	5/7	2/21	–	–	–
E. coli	–	6/7	8/21	–	–	–
Pseudomonas spp.	5/6	–	–	–	–	–
Pseudomonas aeruginosa	–	–	–	2/3	–	–
Yeast	4/6	5/7	12/21	–	2/3	–

The liver, a major organ involved in metabolic processes, is considered to be one of the most eloquent witness of any disturbance in the body, as it is the subject to different types of etiologic attacks: infectious, toxic, metabolic, nutritional and traumatic [1].

In particular, poultry liver consumption needs a special attention. Indeed, poultry liver is considered to be an important source of nutrients, such as vitamins, macro elements and microelements, in some countries, it is used in pregnant women diet and in nutritional disorders.

The pathogenic and conditional pathogenic microorganisms have endogenous origin, intestinal and urinary tractus bacteria distributed at the tissue level which produced inflammatory mediators and activated heterophils.

In general, broiler livers and spleens are contaminated with microorganisms with intestinal origin. Exposure to stress factors produces the permeability of enteric epithelia, penetration of bacteria into mucosa and colonization of spleen and liver using blood flow [3,4].

Yeasts were isolated from the majority of the samples; probably they have exogenous origin coming from the slaughtering process, manipulation and transport of samples.

Conclusions

Liver, spleen and kidney were the organs with pathogenic and conditional pathogenic microorganisms' contamination, while the heart was proofed to be the safeties organ.

Yeasts were the most prevalent contaminants, probably, coming from the manipulation and transport of the samples.

The classical microbiological exam, using selective culture media, represented a good method to determinate the associated microflora in all types of organs.

The pathogenic and conditional pathogenic microorganisms have endogenous origin, intestinal and urinary tractus bacteria distributed at the tissue level which produced inflammatory mediators and activated heterophils.

This study is part of the POSDRU project 88/1.5/S/52614 "Doctoral scholarships for high quality training for young researchers in the field of agronomy and veterinary medicine" and it is part of the PhD thesis "Correlations between liver pathology in broiler chickens and food safety"-Oana-Mărgărita Ghimpeţeanu.

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The Study of Culturable Micro-organisms in Poultry Slaughterhouse

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Introduction

The aim of the study was to investigate concentration of airborne and environmental micro-organisms and the antibiotic resistant *Escherichia coli* strains isolated in slaughter house as a possible source for poultry meat contamination. Microbial contamination of poultry meat is affected by breeding conditions, feeding, manipulation before slaughtering, slaughter treatment hygiene, slaughterhouse hygiene and worker's hygiene. Slaughterhouse is usually divided into clean and dirty zone, which minimizes contamination of final products, ensures continuous technical processes and material flow. The dirty zone includes shackling by feet and the section for poultry carcasses after electrical immobilization. The dead birds are scaled with water in a closed tunnel. The clean zone consists of evisceration section, water-chilling, cutting, de-boning and packaging section. Micro-organisms found in slaughtered poultry, originate from two main sources: the environment of the slaughterhouse (live poultry, equipment, staff) and the digestive tract of the animals [10]. Shackling, killing and evisceration of poultry contribute to the highest contamination with coliforms. Extended spectrum betalactamases (ESBLs) were phenotypically detected in 43% *E. coli* from surface swabs. PCR analysis revealed the presence of CMY-2 genes.

Material and methods

Three samplings were made at different times in winter from one Slovak poultry slaughterhouse. We collected five samples of bioaerosol and five swabs (only for qualitative estimation) from every section of the processing plant.

Bioaerosols were collected by means of a sampler MAS-100 Eco. The MAS-100 Eco air monitoring system is a compact sampler intended for use with standard Petri dishes with nutrient agars. Petri dishes with Endo agar were placed on top of the dish support of the sampler and after aspiration of a preset volume of air, they were incubated at 37℃. The plate counts were recalculated per 1 m^3 of air. Surface swabs were resuscitated in buffered peptone water and then subcultured on Mac Conkey agar.

Susceptibility (MIC) was determined by colorimetric broth microdilution method according to CLSI guidelines [3] using ampicillin, ampicillin and sulbactam, ceftiofur, ceftriaxon, ceftazidime, ceftazidime and clavulanic acid, gentamicin, streptomycin, neomycin, spectinomycin, nalidixic acid, enrofloxacin, ciprofloxacin, chloramphenicol, florfenicol, tetracycline and cotrimoxazol. Phenotype interpretation of mechanisms of β-lactamases (ESBLs) were based on β-lactams (AMP, A + IB, CTR, CAZ, CAC) MIC levels. ESBL genes for CTX-M and CMY-2 were determined by PCR.

Results and discussion

The highest airborne coliform levels were detected during shackling, killing and evisceration of poultry. *E. coli* were resistant only to ampicillin, tetracyclin or enrofloxacin, without ESBLs (extended spectrum betalactamases) production. However, we found *E. coli* with ESBLs and associated with resistance to quinolones (nalidixic acid, ciprofloxacin and enrofloxacin), streptomycin, tetracyclineand cotrimoxazol in swabs from many surfaces in all parts of poultry slaughterhouse (Table 1).

During scalding internal contamination of the carcasses of broilers can occur through feather follicles [1]. The water-chilling system has been criticized, based on the fact that bacteria can be transferred from one chicken to another through water [5]. Poultry may be contaminated by intestinal bacteria during processing. The respective micro-organisms spread easily from one carcass to another during defeathering and evisceration [7]. Adequate equipment for cleaning and disinfection of hands and tools must be supplied in workrooms; such equipment must be as close as possible to the workstations and water taps must not be hand-operable; these facilities must have hot and cold running water, cleaning and disinfecting products and disposable hand towels to cleanse the instruments. Temperature in the cutting room and packaging room must be controlled at around 12 to 15℃ [5].

Betalactam resistance in the 48 *Escherichia coli* isolates recovered from poultry slaughterhouse reached

89% resistance to ampicillin, 62% resistance to ceftiofur and 22% resistance to cefquinome, while the resistance to ampicillin with sulbactam was only 6%. Resistance to streptomycin and gentamicin was detected in 43% vs. 14% isolates; to tetracycline 33%; to chloramphenicol and florfenicol in 10% vs. 18% isolates; to cotrimoxazol in 35% isolates; to enrofloxacin in 43% isolates. The higher MIC of ceftazidime (3.6 $mg \cdot l^{-1}$) and ceftriaxon (5.2 $mg \cdot l^{-1}$) revealed the presence of ESBLs in 43% of isolates. From the 19 selected phenotypically ESBL positive strains sixteen contained CMY-2 genes while CTX-M genes were not detected by PCR.

Table 1 Concentration of airborne coliforms and *E. coli* resistance in surface swabs in poultry slaughterhouse

The place of sampling	Airborne coliforms (cfu/m^3)	*Escherichia coli* resistance
	1st sampling	
Portioning room	0.75×10^3	ESBL, TET, STM, CMP, FLO, NAL, ENR, COT
Packaging room	0.9×10^2	not isolated
Eviscerating room	2.6×10^4	ESBL, TET, STM, NAL, ENR, CIP, COT
Killing room	2.5×10^4	ESBL, STM, NAL, COT
Shackling room	2.07×10^4	ESBL, STM, NAL, ENR, COT
	2nd sampling	
Portioning room	1.0×10^2	not isolated
Packaging room	2×10^2	ESBL, STM, GEN, NAL, CIP. ENR, CMP, COT
Eviscerating room	0.4×10^3	ESBL, STM, NAL, CIP,ENR,FLO, TET, COT
Killing room	1.8×10^4	ESBL, STM, NAL, CIP,ENR,FLO, TET, COT
Shackling room	5.9×10^4	ESBL, NAL, CIP, ENR
	3rd sampling	
Portioning room	0.5×10^2	only ESBLs
Packaging room	0.5×10^2	not isolated
Eviscerating room	0.4×10^3	only ESBLs
Killing room	1.0×10^2	only ESBLs
Shackling room	1.0×10^2	only ESBLs

Conclusions

High level resistance to tetracycline (46%) was observed in *E. faecalis* isolated from rectal swabs in a Slovak poultry setting [2] which is in agreement with our *E. coli* observations. Our results showed that the source of ESBLs for meat *E. coli* could be environmental microbes from poultry slaughterhouse. The enrofloxacin and ciprofloxacin resistance associated with APEC virulence factors in *E. coli* was detected also in poultry faecal strains in Slovakia [4, 6]. It has been widely argued that the meat products are cooked and therefore it is unlikely that the antibiotic-resistant bacteria present in the raw material canl colonize the human gut. This view is challenged by the study which clearly demonstrated the colonization of humans by antibiotic-resistant *E. coli* in the course of preparing and eating cooked chicken at home [9].

Poultry slaughterhouse employees are exposed to high concentrations of airborne micro-organisms throughout the working hours, that can cause serious diseases. Was found an identical clones of antibiotic-resistant *E. coli* in tested animals faeces and human samples.

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Influence of Neck Bar Placing and Brisket Board on Hygiene in Cubicles with Weaned Beef Bull Calves

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Summary: Two features of equipment, neck bar and brisket board, was used with the purpose to control the position and hygiene of cubicle surface for growing newly weaned bull calves of about 300 kg live weight. The bull calves were accommodated in cubicles dimensioned according to the size of the finishing bulls before being sent to slaughter. Both the neck bar and the brisket board were placed in order to provide an adequate lying length in the cubicle. Hygiene of the cubicle surface was linear scored for the surface covered with urine and dispersed manure separately. Recordings of dung piles were done by the placing, present, back half, front half or present in both halves of the cubicle, by recording numbers of dung piles of about 10 cm in diameter. Results indicate some positive influence of the brisket board but also, as lot of dung were placed in the front on the cubicle surface, calves often rested with their heads facing the alley for reasons not fully understood.

Introduction

The design of the cubicles affects the hygiene, welfare and work consumption as well as work safety of cattle [1,2]. Accommodation of growing young cattle in cubicles normally involves the moving of the animals to larger dimensioned cubicles as the animals grow in size. However, for seasonally born calves, winter housing in cubicles will be a certain challenge as the dimensions of the cubicles has to fit the finishing animal. The cubicles have to accommodate animals from approximately 300 kg live weight up to over 600 kg. The hygiene of the lying area surface of cubicles, dimensioned to host animals of 600 kg, was investigated for identical designed cubicles except for manipulations in the front part of the cubicles with either a neck bar or a brisket board placed to allow an average sized calf a suitable lying area length.

Material and methods

Thirty-six newly weaned bull calves (mean live weight 306 kg, SD 43) of the Charolais breed were allotted to two groups of 18 individuals each. The animals were subjected to identical cubicles (size 2120 mm × 1150 mm) except for the placing of the neck bar and the presence of a brisket board during a period of 24 days. In the NECK BAR group, the neck bar was placed 1330 mm from the curb. In the BRISKET BOARD group, a brisket board was placed about 1330 mm from the curb. Both the neck bar and the brisket board were placed in order to provide an adequate lying length in the cubicle. The brisket board was built by a 50 mm high wood stud with a 50 mm metal pipe on top. The total height was thus 100 mm.

Hygiene of the cubicle was done by linear scoring of the surface covered with urine and manure separately (0 – 100). Recordings of dung piles were done by the placing, present, back half, front half or present in both halves of the cubicle, by recording numbers of dung piles of about 10 cm in diameter. Included were also smaller piles of dung, recorded as parts of a full pile of dung, e. g. 0. 5 or larger, e. g. 2. 5. 10 recording were done during the 24 day experimental period.

The data from the scorings of hygiene in cubicles and animals was compiled and comparisons was done using a GLM model (MiniTab) with the following model:

$$Y = \mu + A_i + B_j + C_k + E_{ijk}$$

Where:

Y was the overall mean
μ was the mean
A_i was the effect of the treatment
B_j was the effect of cubicle number
C_k was the effect of day of recording
E_{ijk} was the residual

The frequency of the placing of dung piles in the cubicles was analysed using Chi-square in MiniTab.

Results and discussion

The contamination of dung in the cubicles was significantly higher for NECK BAR (LS means 1. 3 SE 0. 07 than BRISKET BOARD (LS means 1. 0 SE 0. 10). There was no difference between treatments in surface covered with manure, but the area covered with liquids, was higher in NECK BAR (LS means 8 SE 0. 8) than in the BRISKET BOARD (LS means 4 SE 0. 8. (Table 1)

Table 1 Hygiene scores on dung piles, dispersed dung and liquids on cubicle surfaces using neck bar or brisket board. LS means ± SE

	Dung piles	Dispersed dung	Liquids
NECK BAR	$1.3^{a} \pm 0.07$	29 ± 1.3	$8^{a} \pm 0.8$
BRICKET	$1.0^{b} \pm 0.08$	29 ± 1.4	$4^{b} \pm 0.8$

Columns with different superscript differ significantly ($P < 0.001$)

The placing of dung piles in the back and front of the cubicle floor surface is shown in Fig. 1. It was obvious that the calves turned around in the cubicle and rested with their heads facing the alley. This unwanted lying and also how the calves turned around were observed. The reason for this preference of lying position is obscure but is suggested to be related with social control and ease of avoidance of aggression. Anyhow, hygiene of the cubicle surface was worsened by this, than if the animals rested in the intended direction.

Conclusion

The brisket board seemed to partly control the hygiene of the cubicles more efficiently than the neck bar although both were placed at the same distance from the curb. But as a lot of dung was placed in the front of the cubicle the calves turned around in the cubicle and thus rested with their heads facing the alley.

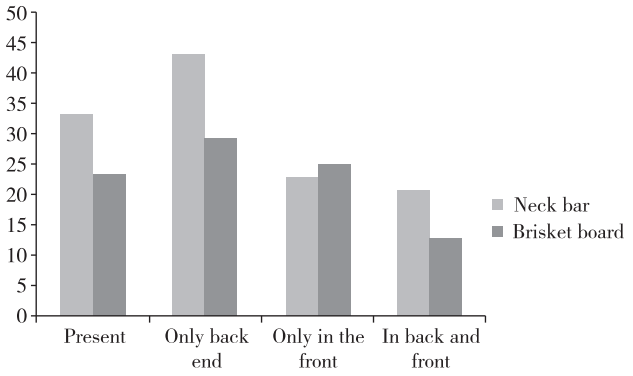

Fig. 1 Piles of dung present in the cubicle, found in only the back end, only in the front half and in both the front and back of the cubicles with either a body length adjusted neck bar or brisket board

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Bacterial Flora of Cultured Catfish Fed with Poultry Hatchery Waste from Selected Farms in Ibadan

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Abstract: Hatchery waste comprises empty shells, infertile eggs, dead embryos, late hatchings, dead chickens, a viscows liquid from eggs and decaying tissue which is used as a cheap fish feed by some farmers. From these animal based feedstuffs, there is a possibility of hazard arising from the presence of viable and contaminating microorganisms.

Microbial quality of poultry hatchery wastes from three selected commercial poultry hatchery units and catfish (*Clarias gariepinus*) fed hatchery waste obtained from five purposively selected aquaculture farms in three local government areas in Ibadan Southwest Nigeria were studied using standard microbiological methods. The result obtained were subjected to statistical analysis.

The total bacterial count obtained ranged from 1.2×10^5 to 4.6×10^6 cfu/g and 1.2×10^5 to 5.6×10^5 cfu/g for the hatchery waste and catfish respectively. The total enterobacterial count ranged from 6.0×1.4 to 3.0×10^5 cfu/g and 4.0×10^4 to 3.0×10^5 cfu/g for hatchery waste and catfish respectively. The Bacteria isolated from hatchery waste were staplylococcus epidermidis, *Esherichia coli*, *Bacillus* spp., *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, while those isolated from catfish organs (skin, stomach and intestines) are *Salmonella* sub sppl, *Leclercia adecarboxylata*, *Bacillus* spp., *Klebsiella pneumoniae*, *Eschericia coli* and *Staphylococcus aureus*, *Citrobacter* spp., *Pseudomonas aeruginosa*. *Salmonella arizonae* sub spp3A.

The types of bacterial organisms that are associated with the hatchery waste and catfish fed hatchery waste found in this study call for concern.

The Influence of Milk Fat Free Fatty Acids Modification on the Quality and Storage Stability of Butter

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Abstract: The aim of the conducted research was the analysis of butter produced of milk fat of dairy cattle yielding 8000 L of milk/year. Butter was manufactured from milk taken from control and experimental groups of multiparous and primaparous cows. Animals were fed with full portion TMR mixed fodder. The feeding dose for the experimental group was enriched with 2% addition of fish oil calculated per fodder's dry matter. The preparation composition was: 25% of fish oil (herring-sprat oil), 33% of beidelit, 33% of vernimiculite and 9% of humocarbowit. Milk used for butter production was taken after 0, 4 and 8 weeks of feeding. The influence of milk fat free fatty acid modification on the quality and storage stability of butter was monitored by the determination of it's basic chemical composition and by the oxidative and hydrolytic changes in butter fat. The hydrolytic changes were analyzed by the determination of acidity and concentration of free fatty acids with the use of gass chromatograph combined with mass spectrometer (GC/MS). The oxydative changes by determination of peroxide number and the TBA factor.

The obtained results shows that the addition of fish oil to the fodder changes the composition of milk fat FFA, increases the amount of nonsaturated FFA conjugated diens of linoleic acid (CLA), eicozapentanoic acid (EPA) and docosahexsaenioc acid (DHA) and lowers the amounts of saturated FFA. The modification of milk fat lowered it's stability by intensification of hydrolytic and oxidative changes. The produced butter was a low quality product showing unacceptable flavor changes.

The Investigation of Ripening Process and Quality Analysis of Cheeses Produced of Milk with the Modified Free Fatty Acids Composition

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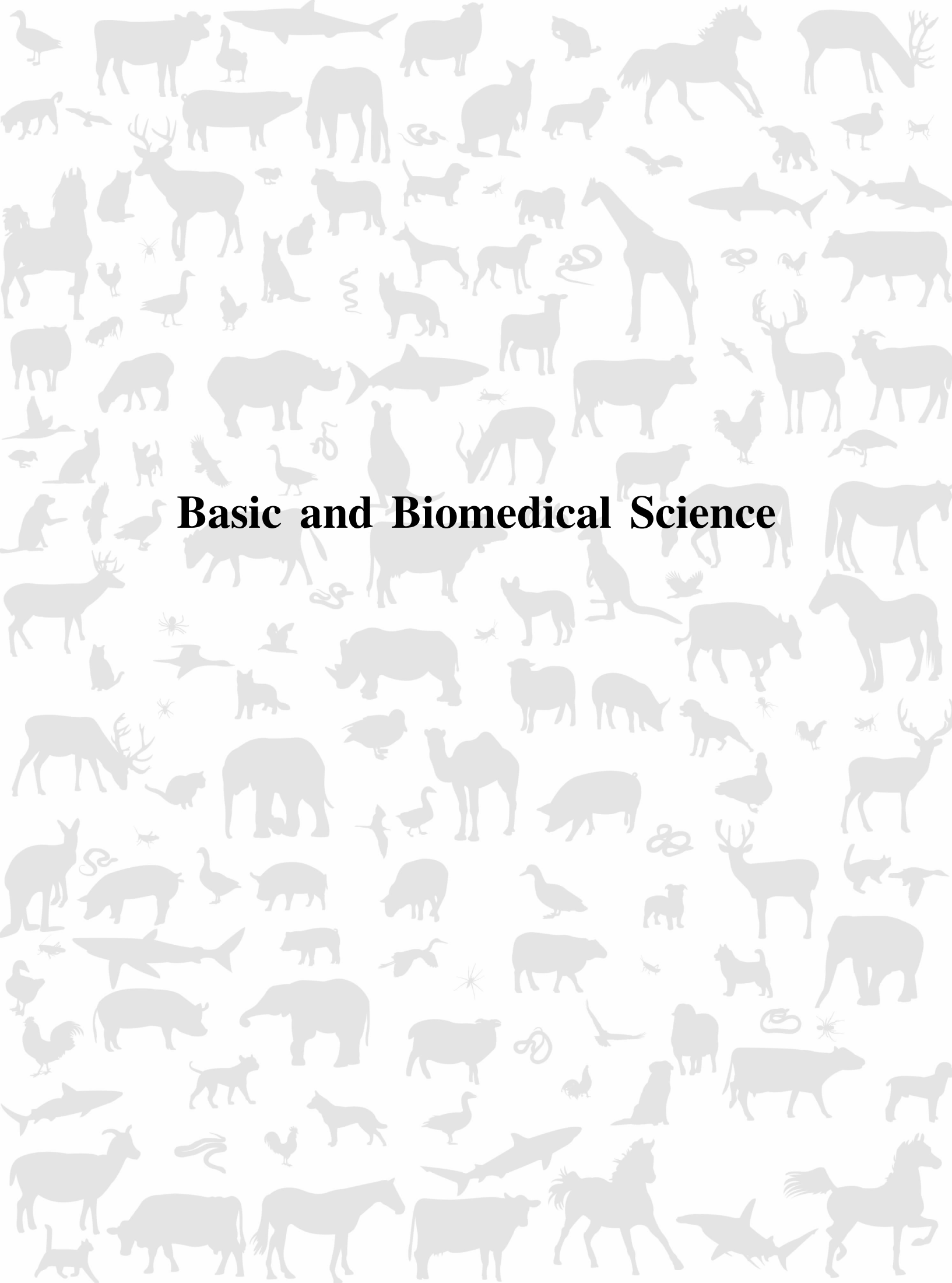
Abstract: The aim of the study was the investigation of ripening process of dutch type, model cheeses produced of milk of high-yielding dairy cows. The feeding dose for the experimental group was enriched with 2% addition of fish oil calculated per fodder's dry matter. Milk used for cheeses production was taken after 0, 4 and 8 weeks of feeding. During the ripening process the changes in the basic chemical composition of cheeses, their acidity and the growth of chosen microbial groups were analyzed to determine the influence of free fatty acid modification on the cheeses quality. The proteolytic changes were monitored by the determination of the soluble nitrogen increase, expressed as the % of total nitrogen as well as by the concentration of free amino groups soluble in water and in phosphotungstic acid. The lipolysis was analyzed on GS/MS and expressed as the increase of FFA concentration. The produced cheeses were subjected to organoleptic analysis.

It was shown that the use of milk with modified composition of FFA did not influenced the yield of produced cheeses and the changes in the determined mesophilic Lactococci, Lactobacilli and yeasts. The estimated values in these analysis, performed during 8 weeks of cheeses ripening, were comparable between the experimental and control groups. The analysis of ripe, 8-week cheeses produced of milk taken at the beginning of the animal feeding showed, that the ratio of soluble to total nitrogen was lower in comparison to values obtained for cheeses manufactured of milk taken after 4 and 8 weeks of feeding with fish oil-enriched fodder. The same observation were made in the free amino groups analysis. The were no significant differences in lipolytic changes between control and experimental cheeses. The modification of milk by the fish oil fodder enrichment did not influenced the sensoric evaluation of analyzed products.



Basic and Biomedical Science

Kidney Affection in Relation to Long Term Administration of Chloramphenicol Succinate

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Summary: This study was designed to investigate the dose and time effects of chloramphenicol administration on the kidneys of animals. Solutions of chloramphenicol succinate were diluted in distilled water and administered to two groups of rats in a dose of 25 and 50 mg/kg body weight to the first and second groups respectively. Another two groups of rats were given distilled and used as control. Rats were scarified under anesthesia after 2, 4, 6, 8, 10 and 12 weeks post dosing. Blood was taken for determination of serum uric acid and creatinine. Histopathological examination of the kidneys was done. Results of uric acid and creatinine showed a significant increase in the used doses of chloramphenicol. This increase could be attributed to renal damage and decrease in glomerular filtration. Histopathological findings of kidney tissues supported that and showed degenerative and necrotic changes in the renal tubules in form of acute hypercelluarity of glomerular tuft, congestion and atrophy of renal tubules. Detectable lesions varied greatly in its severity and distribution according to the length of the period of exposure and the dose of chloramphenicol. A prominent variable sized and shape of mesangial cells of the glomeruli could be observed. Numerous variable sized and shaped electron dense inclusions seems to be fragments of red blood cells engulfed by tubular epithelium. In late stages, changes were represented by necrosis of the renal tubular epithelium with casts in its lumen, hypercelluarity of the glomerular tuft with increase thickness of glomeruli matrix. Differences between the two doses of chloramphenicol were the time and severity degree of appearance of these changes. Toxic changes appeared earlier in higher dose. The variable administered dose of chloramphenicol may play a significant role in its toxicity. This data provoke that chloramphenicol induced nephrotoxic effect in a manner of dose and time related.

Introduction

Veterinary drugs are widely used in domestic animals for prevention and treatment of infectious diseases. However, inappropriate use and abuse of these drugs may bring risk of serious affection, drug resistance and even acute or chronic toxic or allergic reactions by eating the editable tissues of poultry or livestock previously treated with such drugs. Veterinary drug residues in animal products as meats and milk are much more risky especially in developing countries. Chloramphenicol is a potent and broad-spectrum antibiotics used in veterinary practice, including treatment of livestock for several decades (Ramos et al., 2003). Most symptoms are always characterized in dysbacteriosis, agranulocytosis disease, grey baby syndrome and aplastic anemia, which has resulted to its limited usage (Kasten, 2005). Recently some honeys on the international market have been found contaminated with chloramphenicol residues (Rodziewicz and Zawadzka, 2007). Therefore the EU prohibits the use of chloramphenicol as a veterinary drug for food producing animals. Chloramphenicol is still used extensively in many parts of the developing world in human and veterinary medicine either as a therapy against many diseased conditions or as a growth promoter administered in subtherapeutic dose for long period (Stolker and Brinkman, 2005). There are great concerns regarding the human consumption of food products contaminated with drug residues especially in developing countries (Kanarat et al., 2008).

Nephrotoxic effect of chloramphenicol was not fully studied (Abd El-Nasser et al., 2012). The aim of this study was to investigate the possible risk and serious adverse effects attributable to chloramphenicol administration in for twelve weeks on kidney enzymatic activities and histopathological analysis. The selected organ; kidney is important organ in the body because of its involvement in excretion of xenobiotic as extramyeloid origin.

Material and methods

Total of 240 male albino rats weighted 150 – 200 g were divided into four equal groups. Chloramphenicol was administered in a dose of 25 and 50 mg/kg body weight to the first and second groups respectively. Rats in the third and fourth groups were used as control.

Solutions of chloramphenicol succinate (CAPS; Sigma Chemical Co. Ltd, Poole, Dorset, UK) in distilled water were administered to rats with the aid of

special stainless steel gavage. Control animals were given distilled water.

Rats were scarified under anesthesia after 2, 4, 6, 8, 10 and 12 weeks post dosing and whole blood was collected and serum was harvested and stored at –20℃. Creatinine and uric acid concentrations were estimated according to Sies et al. (1985) and Trivedi, et al., (1978) respectively. Kidney samples were taken and fixed in 5% cold buffer glutradehyde for histopathological studies (Gupta, 1983). Samples were processed; sectioned and photographed using Olympus microscope and the ultrathin section were examined and photographed by Jeol 100 CXII transmission electron microscope. Data were analyzed using one-way analysis of Variance (ANOVA) with statistical significance $P < 0.05$ (Oyeka, 1996).

Results and discussion

The obtained results showed significant increase creatinine and uric acid after 8 weeks of exposure and continued till the end of the experiment as presented in Table 1. Histopathological affections of the kidney tissues were summarized in Figures 1 – 12. Hydropic degeneration in the tubular epithelium of moderate degree could be observed with congestion of the capillary tuft of the glomerulus as well as in the inter-tubular blood vessels was recorded. Also slightly increase in the mesangial matrix and thickening of Bowman's capsule could be noticed. Ultrastructurally, the tubular epithelium showed the nucleus situated at the tip of the cell surrounded by wide light electron dense areas and having variable sized and shaped mitochondria.

Table 1 The effect of chloramphenicol on uric acid and creatinine in prophylactic and therapeutic dose rat groups

Groups		Prophylactic dose-treated rat group		Therapeutic dose-treated rat group	
Week	Parameter	Uric acid (mg/dl)	Cratinine (mg/dl)	Uric acid (mg/dl)	Cratinine (mg/dl)
2nd	Dosed	11.24 ± 1.99	0.71 ± 0.058	09.81 ± 2.31	0.68 ± 0.054
	Control	10.99 ± 2.01	0.69 ± 0.099	11.01 ± 1.92	0.74 ± 0.045
4th	Dosed	13.26 ± 1.45	0.84 ± 0.064	12.99 ± 2.01	0.77 ± 0.149
	Control	11.22 ± 1.98	0.72 ± 0.055	10.28 ± 2.13	0.69 ± 0.061
6th	Dosed	11.26 ± 2.22	0.66 ± 0.54	12.43 ± 1.98 *	0.87 ± 0.048 *
	Control	14.25 ± 1.96	0.73 ± 0.06	09.81 ± 2.31	0.67 ± 0.052
8th	Dosed	12.13 ± 1.16	0.81 ± 0.053	15.21 ± 2.08 *	0.91 ± 0.054 * *
	Control	12.99 ± 2.16	0.71 ± 0.049	11.42 ± 2.16	0.70 ± 0.050
10th	Dosed	12.22 ± 2.01	0.89 ± 0.039 *	15.42 ± 1.88 *	0.89 ± 0.039 * *
	Control	11.64 ± 1.94	0.70 ± 0.041	10.59 ± 2.14	0.69 ± 0.056
12th	Dosed	17.22 ± 1.14 *	0.94 ± 0.052 *	19.41 ± 1.88 * *	0.97 ± 0.044 * *
	Control	11.15 ± 1.61	0.69 ± 0.044	12.15 ± 1.59	0.71 ± 0.053

*, * * Significant difference between the control and treated groups ($P < 0.05$ and $P < 0.01$ respectively)

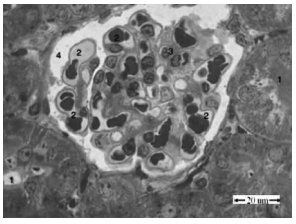

Fig. 1 Semithin section of kidney of group one after six weeks of experiment showing degenerative changes in the epithelium of the kidney tubules (1), congestion of the capillary tuft (2), mild increase in the mesangial matrix (3) with thickening of the bowman's capsule surrounding the bowman's space (4). Toluidine blue stain.

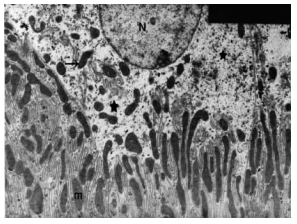

Fig. 2 Transmission electron micrograph of tubular epithelium of group one at the end of the sixth week showing the nucleus of the cell is pushed toward the surface (N), the mitochondria surrounding the nucleus became electron dense (arrow) while the others (m) appears normal. Mag. 8800 ×.

There is clear evidence that, with the increase in consumption of antimicrobial agents by humans or animals, there is a resultant increase in antimicrobial resistance (Donabedian et al., 2003). Various veterinary drugs are widely used and are still using in domestic animals for growth promotion, prevention and treatment of infectious diseases. Chloramphenicol is a potent and broad-spectrum antibiotics used in veterinary practice, including treatment of aquaculture species and livestock husbandry (Liu, et al., 2009). Certain uses of antibiotics in food producing animals can lead to antibiotic resistance in intestinal bacteria, and this

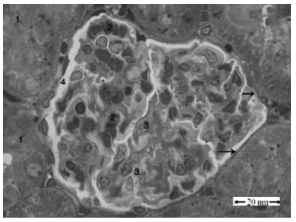

Fig. 3 Semithin section of kidney of group one after ten weeks of the experiment showing congestion of the capillary tuft (2), increase of the mesangial cells (3), narrowing of the bowman's space (4), thickening of the bowman's capsule (arrows) and degenerative changes in the tubular epithelium with presence of deeply stained inclusions in its epithelium (1). Toluidine blue stain.

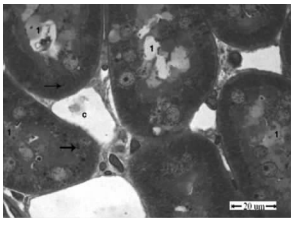

Fig. 5 Semithin section of kidney of group one after twelve weeks of the experiment showing degenerative changes of the tubular epithelium with presence of casts in its lumen (1) also presence of deeply stained inclusions in the tubular epithelium (arrows). Note dilatation of capillaries. Toluidine blue stain.

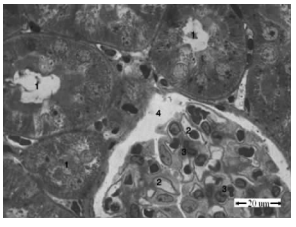

Fig. 7 Semithin section of kidney of group two after four weeks of the experiment showing moderate degree of hydropic degeneration of the tubular epithelium (1), congestion of the capillary tuft (2), as well as inter-tubular vasculature. Moderate increase in the mesangial matrix (3) and thickening of the bowman's capsule surrounding the bowman's space (4). Toluidine blue stain.

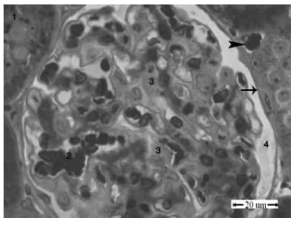

Fig. 9 Semithin section of kidney of group two after six weeks of the experiment showing increase in mesangial matrix (3), congestion of capillary tuft (2), congestion of inter-tubular vasculature (arrowhead), degeneration of the tubular epithelium (1). Toluidine blue stain.

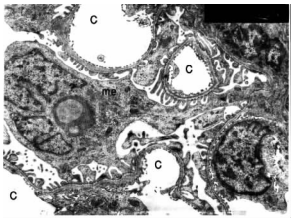

Fig. 4 Transmission electron micrograph of the glumeruli of group one at the end of the tenth week showing increase in mesangial cell and matrix (me). Note the lumen of the capillary tuft (C). Mag. 8800 ×.

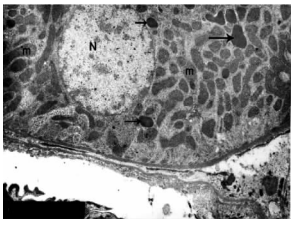

Fig. 6 Transmission electron micrograph of tubular epithelium of group one at the end of the twelve week showing presence of electron dense variable sized fragments (arrows) of red blood cells. Note the nucleus of the epithelium (N) and mitochondria (m). Mag. 8800 ×.

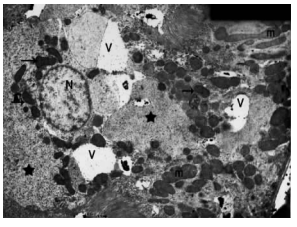

Fig. 8 Transmission electron micrograph of tubular epithelium of group two after 4 weeks showing the epithelial cells greatly swollen ruptured in the lumen of the tubules forming casts. Note vacuoles (V), light electron dense materials (stars), mitochondria (arrows) became electron dense surrounding the nucleus (N) of the ruptured cells while the other mitochondria (m) still normal. Mag. 8800 ×.

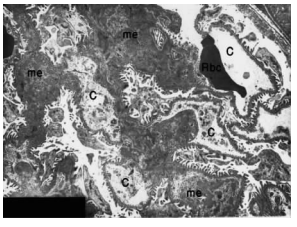

Fig. 10 Transmission electron micrograph of the glumeruli of group two at the end of the eighth week showing marked increase in the mesangial cell and matrix (me). Note capillary tuft (C) contain red blood cell (Rbc). Mag. 6000 ×.

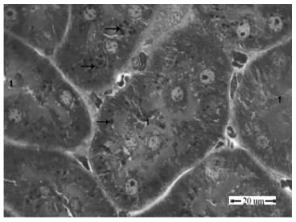

Fig. 11 Semithin section of kidney of group two after ten weeks of the experiment showing marked swelling of the tubular epithelium which lead to obstruction of the lumen (1) with presence of a numerous deeply stained inclusions in the tubular epithelium (arrows). Toluidine blue stain.

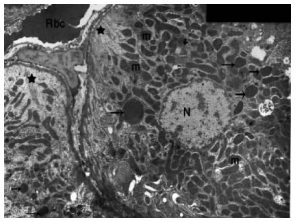

Fig. 12 Transmission electron micrograph of the renal tubular epithelium from group two at the end of the tenth week showing presence of electron dense fragments (arrows) of red blood cells (Rbcs), corrugation of the nucleus (N) and presence of light electron dense material at the base of the cells (star). Note presence of red blood cells (Rbc) in intertubular capillaries. Mag. 8800 ×.

resistance can then be transmitted to the general population, causing treatment resistant illness. These uses of antibiotics can also create antibiotic resistance in non-pathogenic bacteria, the resistance genes of which can be transferred to disease causing bacteria, resulting in antibiotic-resistant infections for humans (Liu, et al., 2009).

The recorded results in this study of both uric acid and creatinine levels in serum showed a significant increase than the control in both used dose. This increase could be attributed to renal damage, decrease in glomeruli infiltration. This explanation was supported with the histopathological findings of kidney tissues that showed degenerative and necrotic changes in the renal tubules in acute hypercelluarity of glomerular tuft, congestion and atrophy of renal tubules in long-term toxicity.

Histopathological investigation of the kidneys from both groups revealed detectable changes in groups of rats exposed to chloramphenicol than control. These lesions varied greatly in its severity and distribution according to the length of the period of exposure and the dose of chloramphenicol. In the early stages, lesions were hydropic degeneration in the tubular epithelium, congestion of the capillary tuft of the glomerulus, increase in the mesangial matrix and thickening of Bowman's capsule. Nucleus situated at the tip of the cell surrounded by wide light electron dense areas and having variable sized and shaped mitochondria were observed in the renal tubular epithelium. Later on; these lesions involving degeneration and necrosis of the renal tubular epithelium with casts in its lumen as well as presence of deeply stained inclusions in the cytoplasm. A prominent variable sized and shape of mesangial cells of the glomeruli could be observed. Numerous variable sized and shaped electron dense inclusions seems to be fragments of red blood cells engulfed by tubular epithelium. Such renal adverse reactions and lesions of a similar nature were reported by Saba et al. (2000).

The degeneration and necrotic changes in the renal tubular epithelium may be attributed to the direct cytotoxic action of chloramphenicol or through its effect on cytochrome P_{450} or might be secondary to hypoxia which resulted from anemia. The tubular necrosis or degeneration is a primary process, it is an important cause of renal failure, and the principal causes of tubular degeneration and necrosis are eschaemia and nephrotoxin. Presence of proteinous material and hyaline casts in the lumen of the degenerated tubules usually indicate disturbance of glomeruli permeability of that nephron which, lead to nephropathy and hypofunction of kidneys manifested clinically by increase creatinine and uric acid level in the serum.

From histopathological investigation of kidney; it can be concluded that the obtained lesions were greatly varied between the early and late stages of chloramphenicol administration. This variation was time and dose dependent. Early changes include hydropic degeneration of the tubular epithelium along with congestion of the capillary tuft of the glomerulus and increase in the mesangial matrix. In late stages changes were represented by necrosis of the renal tubular epithelium with casts in its lumen, hypercelluarity of the glomerular tuft with increase thickness of glomeruli matrix. Numerous variable sized and shaped fragments of red blood cells engulfed by tubular epithelium. These chronic changes were secondary to the primary changes occurred in the renal tubules and glomeruli. Both primary and secondary changes of nephropathies detected in the experimental rats, resulted in permanent lesions and impairment of the renal function which reflected clinically with increase in the creatinine and uric acid level in the serum. In conclusion, this study revealed that Chloramphenicol can induce several alterations in hematological indices, biochemical and histopathological analysis in either prophylactic or therapeutic treated groups of rat. The obtained difference in our results between the prophylactic and therapeutic dose of chloramphenicol

treated groups is the time of appearance to these changes. It was found that anemia, increase in enzymatic activities and histopathological alterations were appeared earlier in therapeutic dose treated rat than in prophylactic one.

Conclusions

Chloramphenicol induced toxic effect in a manner of time and dose related. Kidney function tests and histopathological findings could be attributed to the direct action or indirect through its bactericidal effect on the microorganisms living in the gastrointestinal tract and the release of endotoxins. Endotoxins may lead to induction of the degenerative change occurred in the kidney. Findings in this study widen the scope of understanding of chloramphenicol induced toxicity and establish any nephrotoxic effect of chloramphenicol.

Chloramphenicol is a potent and potentially toxic drug and should be reserved for serious infections in which less toxic antibiotics are ineffective or contraindicated.

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Both Flagella and F4 Fimbriae Contribute F4ac + Enterotoxigenic *Escherichia coli* Adhering to IPEC-J2 Cells *in vitro*

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Summary: While it is known that bacterial tlagella agella generally contribute to pathogen virulence, the role of flagella in the pathogenesis of F4ac + Enterotoxigenic *Escherichia coli* (ETEC) mediated neonatal and post-weaning diarrhea (PWD) is not currently understood. We targeted the reference C83902 ETEC strain (O8: H19: F4ac + LT + STa + STb +), to construct isogenic mutants in the *fliC*, *motA*, and *faeG* genes. Both the Δ*fliC* and Δ*faeG* mutants had a reduced ability to adhere to porcine intestinal epithelial IPEC-J2 cells. F4 fimbriae expression was significantly down-regulated after deleting *fliC*, which revealed that co-regulation exists between flagella and F4 fimbriae. However, there was no difference in adhesion between the Δ*motA* mutant and its parent strain. These data demonstrate that both flagella and F4 fimbriae are required for efficient F4ac + ETEC adhesion *in vitro*.
Key words: flagella; F4 fimbriae; adherence

Introduction

ETEC is a major cause of diarrhea in neonatal and young pigs, causing significant economic losses, treatment costs, and reduced production efficiency. The key virulence factors of ETEC-mediated diarrhea include (i) adhesins, which mediate bacterial attachment to host enterocytes and initiate *E. coli* colonization and (ii) enterotoxins, which disrupt fluid homeostasis in the host small intestine and cause fluid hyper-secretion. ETEC strains expressing F4 (K88) fimbriae, heat-labile enterotoxin (LT), and heat-stable enterotoxin (ST) are the most prevalent. Previous cell culture studies showed that exclusion of F4 + ETEC from attachment to epithelial cells require repression of both the adhesin and LT[1].

Flagella have been generally regarded as a virulence factor, mainly because of their locomotive properties. As more information has been gathered, it is now known that this organelle participates in many additional processes including adhesion, biofilm formation, virulence factor secretion, and the modulation of the immune system of eukaryotic cells[2-4]. The purpose of this study was to investigate whether flagella in F4 + ETEC have similar functions. We deleted the *fliC* gene (encoding the major flagellin protein), the *motA* gene (encoding the *E. coli* flagella motor), and the *faeG* gene (encoding the major subunit of F4 fimbriae) in the wild-type strain C83902 (O8: H19: F4ac + LT + STa + STb +). Using the undifferentiated piglet jejunum intestinal epithelial cell line IPEC-J2 as an *in vitro* cell model, we demonstrated that the flagellum of F4ac + ETEC is an important virulence factor, acting in concert with F4 fimbriae and type I fimbriae, in adhering to IPEC-J2 cells *in vitro*.

Material and methods

Bacterial strains and culture conditions

E. coli C83902 (O8: F4ac +, LT +, STa +, STb +) parent strain and the isogenic mutants C83902 Δ*fliC*, C83902 Δ*motA*, C83902 Δ*faeG*, and double mutants C83902 Δ*fliC* Δ*faeG* were grown in LB broth or on LB agar plates at 37℃, in the presence of ampicillin (100 μg/ml) or chloramphenicol (34 μg/ml) where appropriate.

Cell line culture conditions

Porcine neonatal jejunal epithelial cell line IPEC-J2 cells were cultured in antibiotic free F12-RPMI1640 (1:1) mixed media (Gibco, NY, USA), supplemented with 10% new-borned calf serum (NCS) (Gibco, NY, USA). Cells were maintained in 75 ml flasks (Corning, NY, USA) at 37℃ in a humidified incubator in an atmosphere of 5% CO_2.

Construction of the isogenic mutants for C83902 *E. coli* and the complemented strain Δ*fliC*/*pfliC*

The isogenic C83902 mutants were generated using the λ-Red recombinase as described previously[5]. Full-length *fliC* gene was amplified by PCR from C83902 genomic DNA to generate a complementation strain. The recombinant pBR322 plasmid was then introduced into the Δ*fliC* mutant.

Transmission electron microscopy and motility assays

Flagella morphology of C83902 strain and isogenic mutants were examined using transmission electron microscopy (TEM). Motility assay was done and determined as described previously [6]. Plates were incubated for 32 h at 37℃ before analysis.

Bacterial adherence and adherence inhibition assays

Fimbriae-or flagella-mediated binding specificity of C83902 strain and various deletion mutants were determined by cells adhesion assay. The procedure was described as previously[7]. Rabbit polyclonal antiserum to F4ac fimbriae (raised in our laboratory) at 1∶40 was co-incubated with the responding bacterial suspension for 30 min at 37℃ (5% CO_2) with gentle agitation prior to addition onto the IPEC-J2 cell monolayer. Bacterial adherence was measured as described earlier for the adherence assay.

RNA extraction and fluorescence quantitative PCR

Total RNA was extracted from various bacterial samples using TRIzol reagent (Invitrogen, NY, USA). cDNA was synthesized by using the PrimeScript® T reagent Kit with gDNA Eraser (Takara Bio, Shiga, JPN) for reverse transcription-PCR. GapA was used as an internal control. Real-time PCR amplification followed the guide of SYBR® remix Ex Taq II (Takara Bio, Shiga, JPN). Assays were performed in quadreplicate with a 7500 Fast Real-Time system (Applied Biosystems). The $2^{-\Delta\Delta CT}$ method was right for processing the relative quantification results.

Statistical analysis

Data were analyzed using Student's t-test for independent samples. Differences were considered significant if $P \leqslant 0.05$.

Results and discussion

C83902 *E. coli* was chosen as the parent strain to construct the *fliC*, *motA* and *faeG* isogenic single mutants, as well as the *fliC*& *faeG* double mutant. Wild-type (WT) C83902 was motile on semi-solid agar, while the C83902 Δ*fliC*, C83902 Δ*fliC* Δ*faeG* and C83902 Δ*motA* mutants were non-motile. Unexpectedly, the Δ*faeG* mutant was more motile than WT. Flagella were not detected in *fliC* deletion mutant and Δ*fliC* Δ*faeG* double mutant by TEM. The complemented strain (C83902 Δ*fliC*/p*fliC*) had restored expression of flagella and motility. The fimbriae deletion mutant (C83902 Δ*faeG*) was confirmed by combined methods of DNA sequencing and an agglutination reaction.

To determine whether the flagellar structure itself possessed an adhesive function or if flagellin expression affected other adhesive structures in F4 ETEC, we deleted the *faeG* gene from the parent strain. The isogenic C83902 Δ*faeG* mutant had a 97% reduction in adherence as compared with the parent strain ($P < 0.05$). And a 90% reduction in adherence was obtained by incubating IPEC-J2 cells with anti-F4ac 1∶40 antibody ($P < 0.05$), demonstrated that F4ac fimbriae provided the major adherence factor. The Δ*fliC* mutant also showed a 20% reduction in the ability to adhere to IPEC-J2 cells ($P < 0.05$). Flagellin expression was sufficient to mediate adherence to the IPEC-J2 cells, as the *fliC* complemented strain was able to restore the adherence ability to 94% of WT levels. By contrast, the adherence ability of the flagellin-expressing but non-motile *motA* mutant was not significantly different from the parent strain.

While deleting either *fliC* or *faeG* decreased ETEC adhesion to IPEC-J2 cells, the capacity of the C83902 Δ*fliC* Δ*faeG* double mutant strain to adhere to IPEC-J2 cells was not significantly different from the Δ*faeG* mutant. To determine whether flagella and fimbriae expression is regulated independently, qRT-PCR was used to quantify the expression of *fliC*, *faeG*, and *fimH* in the parent strain and in the various mutants. The expression of *faeG* and *fimH* in the Δ*fliC* mutant was down regulated by 21.5% and 22.5%, respectively, as compared with their expression in the WT strain. By contrast, genes expression in the Δ*motA* mutant was not significantly different. Otherwise, *fimH* and *fliC* were significantly up-regulated after *faeG* deletion ($P < 0.05$). Thus, C83902 Δ*fliC* Δ*faeG* double mutant strain, which expressing neither F4 fimbriae nor flagella, was on a similar expression level of *fimH* compared with WT strain.

As the mRNA expression of the *faeG* gene decreased 21.5% in the Δ*fliC* mutant compared to the parent strain. We suggest that the 20% reduction of the C83902 Δ*fliC* mutant could be due to the down-regulated expression of F4 fimbriae by the absence of flagellin. qRT-PCR was done to check the mRNA expression of *faeG* and *fimH* in the *fliC* complemented strain C83902 Δ*fliC*/p*fliC* as well. The results shown that the expression of *faeG* and *fimH* recovered to a normal level separately compared with the WT strain (expression of *faeG* and *fimH* in complement strain is about 103% and 98% in average respectively of the WT), which also confirmed our suggestion.

It has also been demonstrated that flagellum-mediated motility is essential for enhancing pathogen-host interactions and for promoting the subsequent adherence and colonization of several gram-negative pathogens[8]. Surprisingly, there were no differences in the mRNA expression level of above-mentioned flagellin or fimbriae between the highly motile *E. coli* C83902 strain and the non-motile *motA* deletion mutant. Even in our adherence assay, the adherence ability of *motA* deletion mutant was the same as the parent strain. These data indicated that

motility may be unnecessary for *E. coli* C83902 strain adherence to IPEC-J2 cells *in vitro*.

This study provides the first evidence that the expression of flagella coordinately regulates the expression of other fimbriae in F4 + ETEC. From our qRT-PCR data, flagella expression is correlated with the expression of F4 fimbriae and type I fimbriae. We observed that deletion of flagellin repressed F4 fimbriae and type I fimbriae expression. Without F4 fimbriae expression, bacteria could express more flagella and typeI fimbriae, which directly enhance motility. As a result, when we deleted both *fliC* and *faeG* genes, the expression level of type I fimbriae returned to a normal level. Loss of type I fimbriae expression due to the decreased expression of flagella was reported already, but the mechanism by which flagellar deficiency alters F4 fimbriae expression is unclear[9]. One possibility is that there may be a regulatory crosstalk among bacterial surface organelles. Further work is needed to study the coordinate regulatory mechanism of these surface structures in F4 + ETEC.

Johnson et al. reported that the heat-labile enterotoxin promotes Escherichia coli adherence to intestinal epithelial cells[1]. Flagellin may regulate some virulence factors such as LT secretion by affecting different types of secretion system[10].

Conclusions

We first demonstrated that flagella of F4ac + ETEC is an non-ignorable virulence factor and its expression had significant correlation with other virulence factors, such as F4 fimbriae and type I fimbriae. Flagellin absence down-regulated the expression of F4 fimbriae and decreased the adhesion to IPEC-J2 cells *in vitro*.

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A Non-coding Small RNA MicC Regulates Virulence and Outer Membrane Proteins Expression in *Salmonella enteritidis*

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Summary: Non-coding small RNA (sRNA) is a new factor to regulate gene expression at post-transcriptional level. MicC is a sRNA existed in *Escherichia coli* and *Salmonella typhimurium* which can repress the expression of outer membrane proteins OmpC and OmpD respectively. In recent years, *Salmonella enteritidis* has become the most prevalent non-typhoidal serovar in many countries. To investigate the regulation function of *micC* in other target genes and virulence for animals, we cloned the *micC* gene in the strain *S. enteritidis* 50336, and then constructed the mutant 50336Δ*micC* by the λ Red-based recombination system and the complemented mutant c50336Δ*micC* carrying recombinant plasmid pBR322 expressing *micC*. Novel target genes were screened by qRT-PCR. The results demonstrated that transcription of *ompD* in 50336Δ*micC* was increased slightly (1.3-fold) than that in wild type strain, while the protein expression of OmpD was increased evidently in 50336Δ*micC*. In addition, the transcription of *ompA* and *ompC* in 50336Δ*micC* increased about 2.2-fold and 3-fold than those in wild type strain. It indicated that *micC* could repress the expression of *ompA* and *ompC*. *OmpA* was probably a novel target genes regulated by *micC* directly. Because OmpA and OmpD were related to adhesion to host cells and mediated virulence finally, the pathogenicity of 50336Δ*micC* was detected by both infecting 6-week-old Balb/c mice by intraperitoneal injection and 1-day-old chickens by hypodermic injection. The result showed that the LD_{50} of the wild type strain 50336, the mutants 50336Δ*micC* and c50336Δ*micC* for 6-week-old Balb/c mice were 12.59 cfu, 5.01 cfu, 19.95 cfu respectively. The LD_{50} for 1-day-old chickens were 1.13×10^9 cfu, 1.55×10^8 cfu, 2.54×10^8 cfu respectively. It indicated that deletion of *micC* enhanced virulence of *S. enteritidis* in mice and chickens by regulating expression of outer membrane proteins, especially OmpA and OmpD.

Introduction

Non-coding small RNAs (sRNAs) are 40 – 400 nucleotides in length, which generally do not encode proteins but could be transcribed independently in bacterial chromosomes [1 – 3]. Most of sRNAs are encoded in the "empty" intergenic regions (IGRs) between gene-coding regions and interact with *trans*-encoded target mRNAs through base-pairing actions, and regulate target genes expression at the post-transcriptional level[4, 5]. They play an important regulation role in substance metabolism, outer membrane proteins synthesis, quorum sensing and virulence gene expression [5]. *MicC* is an about 100-nucleotide small RNA transcript existed in *Escherichia coli* and *Salmonella typhimurium*[6, 7]. MicC could regulate the expression of OmpC by inhibiting ribosome binding to the *ompC* mRNA leader in vitro and it require the Hfq RNA chaperone for its function in *Escherichia coli* [6]. In *Salmonella typhimurium*, MicC could silence *ompD* mRNA via a ≤12-bp RNA duplex within the coding sequence (codons 23 – 26) and then destabilize endonucleolytic mRNA[7]. The OmpC is an abundant outer membrane protein which was thought to be important in environment where nutrient and toxin concentrations are high, such as in the intestine[6]. The OmpD porin is the most abundant outer membrane protein in *Salmonella enterica serovar* Typhimurium and represents about 1% of total cell protein[8]. OmpD is involved in adherence to human macrophages and intestinal epithelial cells[9]. MicC could repress the expression of both OmpC and OmpD porins. It was supposed that MicC maybe regulate virulence. To explore new target genes regulated by MicC and study the virulence regulation function of *micC*, we cloned the *micC* gene in the *Salmonella enteritidis* strain 50336, then constructed the mutant 50336Δ*micC* and the complemented mutant c50336Δ*micC*. Novel target genes were screened by qRT-PCR. The virulence of 50336Δ*micC* were detected by animal infections. We found that MicC could regulate the expression of OmpA and OmpC. The delection of *micC* enhanced virulence of *S. enteritidis* in mice and chickens.

Material and methods

Bacterial strains, plasmids and culture conditions

Salmonella enteritidis strain 50336, the mutant 50336Δ*micC*, complemented mutant c50336Δ*micC* and

E. coli DH5α were grown in LB broth or on LB agar plates at 37℃, in the presence of ampicillin (100 μg/ml) when appropriate. The plasmids pKD46, pKD3 and pCP20 were used for deletion mutant construction. The plasmid pBR322 was used to express MicC in the strain 50336 Δ*micC* for complemented mutant construction.

Construction of the mutant 50336 Δ*micC* and the complemented strain c50336 Δ*micC*

The *micC*-negative mutant of *Salmonella enteritidis* strain 50336 was constructed by the phageλ-Red-mediated recombination system as described previously [10]. Bacterial strains were routinely grown at 37℃ except for strains containing the temperature sensitive plasmids, pKD46 or pCP20, which were grown at 30℃. The chloramphenicol resistance-encoding gene which contained regions homologous to *micC* gene was amplified by PCR amplification. The PCR products were purified and introduced into plasmid pKD46-containing *S. enteritidis* 50336 by electroporation. Recombinant bacteria were screened and selected on both Cm and Amp resistance LB agar plates. The *S. enteritidis micC*::*cat* was used to excise the Cm cassette by introducing the Flp recombinase-expressing vector pCP20. The final mutant 50336 Δ*micC* was confirmed by PCR and DNA sequencing.

To construct the complemented mutant, full-length *micC* gene was amplified by PCR and ligated to plasmid pBR322. The recombined plasmid pBR322-micC was transferred to the mutant 50336 Δ*micC* and then obtained complemented strain c50336 Δ*micC*.

RNA extraction and fluorescence quantitative PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, NY, USA). cDNA was synthesized by using the PrimeScript RRT reagent Kit with gDNA Eraser (Takara Bio, Shiga, JPN) for reverse transcription-PCR. Real-time PCR amplification was performed by the guide of SYBR Premix Ex Taq II (Takara Bio, Shiga, JPN) in triplicate with the ABI7500 instrument (Applied Biosystems). All data were normalized to the endogenous reference gene *gyrA*. The $2^{-\Delta\Delta CT}$ method was used for processing datas.

Animal infections

Bacterial cultures for mice and chicken infections were grown in LB to early stationary phase (OD_{600} of 2 – 3) at 24℃, harvested by centrifugation, and diluted to appropriate cfu ml^{-1} in sterile PBS for infections. For mice infections, *Salmonella enteritidis* wild type strain 50336, mutant 50336Δ*micC* and complemented strain c50336Δ*micC* were resuspended to 10 cfu/200 μl, 10^2 cfu/200 μl and 10^3 cfu/200 μl gradient resuspensions used to infect groups of five 6 – 8 week old Balb/c mice per strain by hypodermic injection. The control group was injected with 200 μl physiological saline. For chicken infections, three strains were resuspended to 10^7 cfu/200 μl, 10^8 cfu/200 μl and 10^9 cfu/200 μl gradient resuspensions used to infect groups of twenty 1-day-old chickens per strain. All the experimental animals were monitored daily for signs of illness and deaths. LD50 (median lethal dose) was calculated by SPSS17 data analysis soft.

Results and discussion

In this study, we cloned the *micC* gene in the strain *S. enteritidis* 50336 based on the sequences of *micC* gene in *S. typhimurium*. Result showed that the sequence of MicC in *S. enteritidis* 50336 was the same as that in *S. typhimurium*. To identify MicC targets in *S. enteritidis* and investigate MicC mediated virulence for animals, we constructed the deletion mutant 50336Δ*micC* by the λ Red-based recombination system and the complemented mutant c50336 Δ*micC* carrying recombinant plasmid pBR322 expressing *micC* successfully. The mutant and its complemented strain were the base to study the function of sRNA *micC* gene. The targets of MicC were identified by real-time quantitative PCR. In our study, the mRNA level of outer membrane protein genes *ompA*, *ompC* and *ompD* were changed in the deletion mutant 50336 Δ*micC*. Compared with *S. enteritidis* 50336 wild type strain, the transcription of *ompD* in 50336 Δ*micC* was increased slightly (1.3-fold) than that in wild type strain, but the protein expression of OmpD was increased evidently. In addition, the mRNA of *ompA* and *ompC* in 50336 Δ*micC* increased about 2.2-fold and 3-fold than those in wild type strain, while recovered in the complemented strain c50336Δ*micC*. It indicated that *micC* could regulate the expression of OmpA and OmpC. Pfeiffer's research found that OmpC and OmpD were the main targets of MicC. MicC could act by accelerating *ompD* mRNA decay but not control translational initiation of *ompD* mRNA [7]. In *E. coli*, MicC was shown to inhibit ribosome binding to the *ompC* mRNA 5' leader [6]. It indicated that MicC could regulate targets by different regulation mechanism. In our study, we found that MicC control the mRNA level of *ompC* and *ompD* in *S. enteritidis*. Moreover, a novel target OmpA was discovered. But the regulation mechanism was not clear and remains to be studied in future. OmpA, OmpC and OmpD are all important and abundant outer membrane proteins. OmpC play an important role in abominable environment such as in the intestine[6]. OmpD is involved in adherence to human macrophages and intestinal epithelial cells[9]. It was supposed that the change of OMPs expression caused by MicC deletion could influence the virulence of *S. enteritidis*. Animal infections experiment showed that the LD_{50} of the wild type strain 50336, the mutants 50336 Δ*micC* and c50336 Δ*micC* for 6-week-old Balb/c mice

were 12.59 cfu, 5.01 cfu, 19.95 cfu respectively. The LD_{50} for 1-day-old chickens were 1.13×10^9 cfu, 1.55×10^8 cfu, 2.54×10^8 cfu respectively. It indicated that deletion of *micC* enhanced virulence of *S. enteritidis* in mice and chickens by regulating expression of outer membrane proteins, especially OmpA and OmpD.

Conclusions

We loned the *micC* gene in the strain *S. enteritidis* 50336 and constructed the deletion mutant 50336Δ*micC* and the complemented mutant c50336Δ*micC* successfully. MicC could regulate the expression of *ompA*, *ompC* and *ompD*. The deletion of MicC could enhance virulence of *S. enteritidis* in mice and chickens. It was supposed that the deletion of MicC led to many virulence related genes changed, eventually, led to virulence alteration.

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Pathogenicity of the *S. enteritidis* with SEf14 Fimbriae Subunit *sefA* and *sefD* Gene Deletion

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Summary: SEF14 fimbriae are only expressed in *S. enteritidis* and closely related *S. enterica serovars*, and by now the role of SEF14 fimbriae in virulence remains to be elucidated. To investigate the survival function of SEF14 fimbraie in animal macrophages and the host itself, SEF14 fimbriae deletion mutants 50336Δ*sefA* and 50336Δ*sefD* established from strain 50336 were thoroughly examined in peritoneal macrophages *in vitro*, as well as in mice and chicks *in vivo*. Results exhibited that both survival numbers of 50336Δ*sefA* and 50336Δ*sefD* were significantly different from strain 50336, whether within a 1.5h's or 3h's interaction ($P < 0.05$). Additionally, subsequent LD_{50} (lethal dose 50%) data collected from 6-week-old Balb/c mice intra-peritoneally injected with 50336, 50336Δ*sefA*, and 50336Δ*sefD* were 19.95, 7.94, and 7.94 cfu, respectively. In *vivo* experiments with chicks, the above three strains exhibited rather different ability in causing death of day-olds. Data also showed that injection with 50336Δ*sefA* or 50336Δ*sefD* each alone had caused reduced weight-gaining in 7-day-olds. This study predicted that *sefA* and *sefD* subunits of SEF14 fimbriae do enhance *S. enteritidis* survival rate in macrophages *in vitro*, and that may enhance the virulence of *S. enteritidis* in 6-week-old Balb/c mice and chicks *in vivo*.

Introduction

S. enteritidis is a facultative intracellular pathogen that can cause severe food-borne infections in humans, mainly due to consumption of chicken meat and egg products [1]. As we know for ages that SEF14 fimbriae were only expressed in *S. enteritidis* and closely related serovars [2], we may imagine they may play a certain serovar-specific role in pathogenesis. To date, just few studies were performed to study the role of SEF14 fimbriae in *S. enteritidis* pathogenic process, and the results sometimes were controversial [3, 4]. In the present study, isogenic mutants *sefD* and *sefA* were constructed by using the combined methods of λ Red recombinase system and suicide vector system to investigate the role of SEF14 fimbriae in mice peritoneal macrophages *in vitro* and in mice as well as chicks *in vivo*, separately.

Material and methods

The reference strain 50336 for *S. enteritidis* was used in this study. The *sefD* gene of *S. enteritidis* 50336 was deleted by homologous recombination of PCR fragment with λ Red recombinase system[5], and the mutant of *sefA* in-frame deletion with 99 bp in strain 50336 was constructed according to the method previously described [6]. The oligo nucleotide sequences of the primers used were showed in Table 1. Survival of *S. enteritidis* and its isogenic mutants in activated mouse peritoneal macrophages was assayed as described previously [7]. We utilized 6-weeks-old Balb/c mice and day-old chicks model to measure the difference of virulence among 50336, 50336Δ*sefA* and 50336Δ*sefD* strains[8]

Table 1 Oligo nucleotide sequences used for PCR primers or sequencing

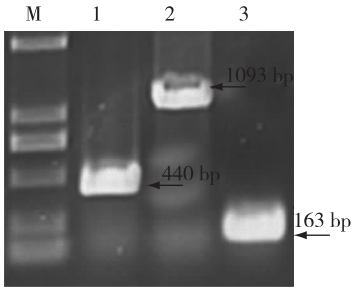
Primer	Sequence 5' – 3'	Size/bp
P1	5'-GAA TCA GTA TAA TTC GTC AAT ACC TAA GTT CAT TGT CTC TGT TTT TCT GAT GTG TAG GCT GGA GCT GCT TCG-3'	1114
P2	5'-ATT CAA TTT CTG TCG CAT ATA TGC TTA TTA AAT ATG TGT CAA CAG GAA CAT ATG AAT ATC CTC CTT AG-3'	
P3	5'-GAA TCA GTA TAA TTC GTC AAT ACC TAA G-3'	440/163
P4	5'-ATT CAA TTT CTG TCG CAT ATA TGC TTA A-3'	
P5	5'-ACT CTA GAC TTT CGC CCG CAG CAC CT-3'	1000
P6	5'-CCA GGA TCC TGA CTC CAG TT-3'	
P7	5'-ATC TCGAGG ATG GAC AAG GAC AGC CTG TT-3'	990
P8	5'-GCG GTA CCT CTT ATA ATT TCA GCG CCG TAA T-3'	
P9	5'-CGC ATA TGG CTG GCT TTG TTG GTA AC-3'	445/346
P0	5'-CGA CTA GTT TAG TTT TGA TAC TGC TGA A-3'	

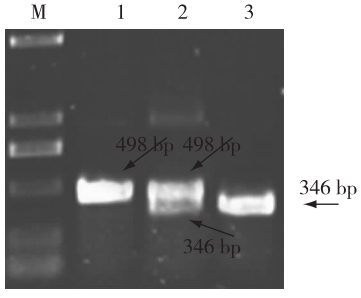
Results and discussion

In the present study, isogenic mutants of *sefA*, *sefD* of SEF14 fimbriae in *S. enteritidis* strain 50336 were successfully constructed using the methods of the suicide vector system and the red recombinase system, results were showed in Fig. 1 and Fig. 2. *S. enteritidis* can enter macrophages by phagocytosis of macrophages (passively for *S. enteritidis*) and by invasion of *S. enteritidis* in these cells (actively by *S. enteritidis*). In this experiment we calculated that *S. enteritidis* 50336 strain and its isogenic mutants 50336Δ*sefA* and 50336Δ*sefD* could enter the macrophages in a short time (0.5 h) after inoculation, and these macrophages also started clearing the bacteriae immediately, the number of bacteriae in macrophages were decreased at 3.5 h. These results were accordance with Kramer reported in 2002[9]. The survival number of 50336Δ*sefA*, 50336Δ*sefD* mutants in mouse peritoneal macrophages was significantly different from strain 50336 at 1.5 h and 3 h interaction ($P < 0.05$), we concluded that 50336Δ*sefA*, 50336Δ*sefD* mutants could survive in macrophages better than wild strain 50336, and the SEF14 fimbriae influenced survival of *S. enteritidis* in macrophages. Further, the significance of gene *sefA* and *sefD* of SEF14 fimbriae in the virulence were investigated in mice by comparing the LD_{50}. The results showed mutants 50336Δ*sefA* and 50336Δ*sefD* were significant different with wild strain 50336 which implying that SEF14 did play a significant role in the virulence of *S. enteritidis* in mice. Further more, we tested the virulence of *S. enteritidis* and its isogenic mutants in day-old chicks. The observation of infection of chicks with strain 50336 and mutant strains 50336Δ*sefA* and 50336Δ*sefD* indicated that they are highly heterogeneous in their ability to cause death in 1-day-chicks, the mortality of 50336 and 50336Δ*sefA* groups were 8.6%, 15% respectively (Table 2). The results also showed that injection with 50336Δ*sefA* reduced the gaining in weight in 7-day-old, gaining in weight of control group was 26.43 g, and 50336 group was 19.06 g, while groups of 50336Δ*sefA* and 50336Δ*sefD* were 15.38 g, 18.39 g respectively (Table 2). These results were in accordance with the LD_{50} assay which showed that gene *sefA* and *sefD* also influenced the virulence of strain 50336 in chicks. Edward reported that polar mutations that disrupt the entire *sef* operon of *S. enteritidis* decreased virulence in mice more than 1,000-fold. Nonpolar mutations that disrupted *sefA* (encoding the major structural subunit) did not affect virulence, but mutations that disrupted *sefD* (encoding the putative adhesion subunit) resulted in a severe virulence defect [3]. These results were controversial with our assays; it could be explain by our using the different *S. enteritidis* strains and different culture method for expressing SEF14 fimbraie in virulence assays. And pathogenicity of SEF14 fimbraie of *S. enteritidis* also needed further study.

Table 2 Mortality rate and weight gaining of challenged chicken (mean ± SD)

Group	Mortality (Death/Total)	Weight gain at 7days(g)
50336	6/70(8.6%)	19.06 ± 8.94
50336Δ*sefA*	3/20(15%)	15.38 ± 9.58
50336Δ*sefD*	0/20	18.39 ± 7.59
Control group	0/20	26.43 ± 3.27





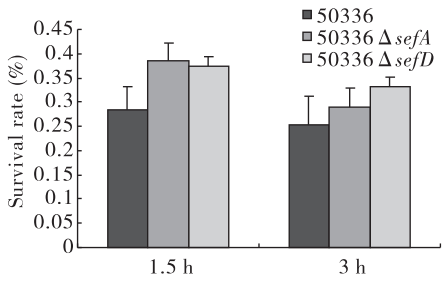


Fig. 1 50336Δ*sefD* isogenic mutants by PCR detection (M. Molecular mass marker DL2000; Lane 1. Wild-type *S. enteritidis* 50336; Lane 2. The 1st recombinant 50336Δ*sefD*::*Cat*; Lane 3. The 2nd recombinant 50336Δ*sefD*)

Fig. 2 50336Δ*sefA* in-frame deletion mutants by PCR detection (M. Molecular mass marker DL2000; Lane 1. Wild type *S. enteritidis* 50336; Lane 2. The 1st recombinant 50336Δ*sefA*::*Cat*; Lane 3. The 2nd recombinant 50336Δ*sefA*)

Fig. 3 Survival and replication of *S. enteritidis* and its isogenic mutants in the activated mice peritoneal macrophages (Survival and replication ability was measured by the survival rate after 1.5 or 3 h of co-incubation with activated mice peritoneal macrophages, which corresponds to (CFU of intracellular bacteria/CFU of input bacteria) × 100. Results from a single representative experiment performed in triplicate are presented.)

Conclusions

We concluded that the SEF14 fimbriae influence survival of *S. enteritidis* in macrophages, and the virulence assays in mice and day-old chicks also showed that gene *sefA* and *sefD* influenced the virulence of strain 50336 *in vivo*, the virulence of 50336Δ*sefA* and 50336Δ*sefD* increased compared with wild strain. Thus, the results indicated that the SEF14 fimbriae may reduce the full virulence of *S. enteritidis*.

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IsrE, a Small Non-coding RNA of Invasion Gene Island (SPI-1) Involved in the Virulence of *Salmonella enteritidis*

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Summary: Small non-coding RNAs (sRNA) of a kind of post-transcriptional regulators in bacteria have excited much interest lately and *Salmonella* has become a model organism for sRNA research. A small non-coding RNA, IsrE, was first identified in pathogenicity island (SPI-1) of *Salmonella typhimurium*. However, the regulation function of IsrE in virulence genes is still unknown. To investigate the regulation function of IsrE in virulence, we cloned the *isrE* gene in the reference strain *S. enteritidis* 50336, and then constructed the inframe deletion mutant 50336Δ*isrE* by the λ-Red-based recombination system. Novel target genes were screened by qRT-PCR. The result showed that the deletion of *isrE* significantly decreased the transcription of both outer membrane protein *ompD* (25-folds) and flagella subunit *fliC* (8.6-folds) compared to those in wild type strain, while the motility of mutant 50336Δ*isrE* was decreased evidently. In addition, the transcription of *fimA*, *sefA*, *csgA*, *csgD* and *sthA* in 50336Δ*isrE* were decreased about 3 to 5 folds. It indicated that IsrE could activate the transcription of *ompD* and *fliC* significantly and facilitates the expression of *fimA*, *sefA*, *csgA*, *csgD* and *sthA* in *S. enteritidis*. The mutant strain 50336Δ*isrE* exhibited an decreased ability to adhere to Caco-2 cells as well as the motility ability compared to the wild type strain. The pathogenicity of 50336Δ*isrE* was detected by infecting 1-day-old chickens by hypodermic injection. The LD_{50} of the wild type strain 50336 and the mutant 50336Δ*isrE* for 1-day-old chickens were 2.809×10^8 cfu and 4.084×10^8 cfu respectively. Our study shows that the deletion of *isrE* may attenuate the virulence of *S. enteritidis* in chickens by regulating expression of outer membrane protein OmpD, flagella FliC and fimbriae subunits.

Introduction

Now, post-transcriptional control at the mRNA level is well accepted to play an important role in bacterial gene expression. To date, small non-coding RNAs (sRNAs) have been found to constitute the largest class of post-transcriptional regulators in bacteria. Most sRNAs range from 40 to 400 nucleotides in length, lack open reading frames and are encoded in the intergenic regions (IGRs) between gene-coding regions[1-3]. By far, the largest group of *Salmonella* sRNAs act by base-pairing at the level of mRNA translation or stability. Depending on their location relative to targets, these base-pairing sRNAs are referred to as 'cis-antisense' or 'trans-antisense'. Trans-antisense sRNAs, each of which might act on several target mRNAs in parallel, seem to constitute the largest class of regulatory sRNAs in *Salmonella* and most tans-encoded sRNAs require the RNA chaperone Hfq for both productive annealing to targets and their own intracellular stability[4]. Base-pairing by trans-antisense sRNAs is usually short and imperfect and the outcome of the regulation is most often negative, leading to decreased stability or translational repression of the target[5]. However, IsrE, an island-encoded sRNA, was found to increase the stability of its several probable target mRNAs in our research. IsrE is an additional Fur-controlled RyhB-like sRNA from the STM1273/yeaQ IGR in *Salmonella*[6], Which was also named RyhB-2[7] or RfrB[8]. *IsrE* shares its own regulation by Fur and its repressor function of sodB mRNA with *RyhB*[8], but these two similar sRNAs show quite different expression patterns during growth and non-redundant activity in the iron stress response of Salmonella[6]. To explore new target genes regulated by IsrE and study its virulence regulation function, we cloned the *isrE* gene in the *Salmonella enteritidis* strain 50336, then constructed the mutant 50336Δ*isrE* and the complemented mutant c50336Δ*isrE*. Novel target genes were screened by qRT-PCR. The virulence of 50336Δ*isrE* were detected by animal infections and bacterial adherence assays. We also evaluate the motility ability of 50336Δ*isrE* by motility assays. We found that IsrE could regulate the expression of outer membrane protein *ompD*, flagella Subunit *flic* and fimbriae subunits *fimA*, *sefA*, *csgA*, *csgD sthA* at the mRNAs level and the deletion of *isrE* decreased the virulence of *S. enteritidis* in chickens and adherence ability.

Materials and methods

Bacterial strains, plasmids and cell culture conditions

Salmonella enteritidis strain 50336, the mutant 50336Δ*isrE*, and *E. coli* DH5α were grown in LB broth or on LB agar plates at 37℃. The plasmids pKD46, pKD3 and pCP20 were used for gene deletion mutant

construction. Human colorectal adenocarcinoma epithelial cells(Caco-2) were cultured in Dulbecco's minimal Eagle medium (DMEM) containing glutamine (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) and maintained at 37℃ and 5% CO_2.

Construction of the mutant 50336Δ*isrE*

The *Salmonella enteritidis* strain 50336Δ*isrE* mutant was constructed by the phage λ-Red-mediated recombination system as described previously [9]. Bacterial strains were routinely grown at 37℃ except for strains containing the temperature sensitive plasmids, pKD46 or pCP20, which were grown at 30℃. The chloramphenicol resistance-encoding gene which contained regions homologous to *isrE* gene was amplified by PCR amplification. The PCR products were purified and introduced into plasmid pKD46-containing *S. enteritidis* 50336 by electroporation. Recombinant bacteria were screened and selected on both Cm and Amp resistance LB agar plates. The *S. enteritidis isrE*::cat was used to excise the Cm cassette by introducing the Flp recombinase-expressing vector pCP20. The final mutant 50336Δ*isrE* was confirmed by combined PCR and DNA sequencing.

RNA extraction and fluorescence quantitative PCR

Both analysis of *isrE* dynamic expression in *Salmonella enteritidis* strain 50336 and screening of novel target genes of IsrE were performed by fluorescence quantitative PCR(qRT-PCR). Total RNA was extracted using TRIzol reagent (Invitrogen, NY, USA). cDNA was synthesized by using the PrimeScript RRT reagent Kit with gDNA Eraser (Takara Bio, Shiga, JPN). Real-time PCR amplification was performed under the guide of SYBR Premix Ex Taq II (Takara Bio, Shiga, JPN) in triplicate with the ABI7500 instrument (Applied Biosystems). All data were normalized to the endogenous reference gene *gyrA*. The $2^{-\Delta\Delta CT}$ method was used for processing datas.

Motility assays

The motility assay was performed on semisolid agar plates as described previously[10]. 50 μl of overnight cultured bacterial stock was re-inoculated into 5 ml of sterile LB broth and incubated at 37℃ with aeration in a bacterial shaker at 178 rpm. IsrE was proved to accumulate to peak at the grown phase when the optical density value at OD_{600} was between 2.5 and 3.0. At this phase culture samples were diluted until the optical density value of approximately 1.0 at OD_{600}. Then 1 μl of the diluted culture samples from the wild type strain and the mutant strain were seeded onto 0.3% tryptone agar plates (Tryptone 1%, NaCl 0.25%, Agar 0.3%). Motility was measured after incubation for about 18 h at 37℃ by observing bacterial growth spread around the initial inoculum as evidenced by enhanced turbidity.

Adherence assays

Bacterial adherence assays was performed as described previously [11]. Briefly, 1×10^8 cfu of bacteria were added to a monolayer of approximately 1×10^6 Caco-2 cells in each well of a 96-well tissue culture plate in triplicate. After 2 h incubation, the cell monolayer was gently washed three times with phosphate buffered saline (PBS, pH 7.2) and lysed with 0.5% Triton X-100 for 30 min. The lysates were diluted with PBS and plated onto LB agar plate for bacterial counting.

Animal infections

Bacterial culture for chicken infections were grown to early stationary phase (approximately OD_{600} of 2.5 – 3.0) at 37℃, harvested by centrifugation and washed one time with PBS. Then *Salmonella enteritidis* strain 50336 and mutant 50336Δ*isrE* were resuspended to 10^7 cfu/200 μl, 10^8 cfu/200 μl and 10^9 cfu/200 μl gradient resuspensions used to infect groups of twenty 1-day-old chickens per strain by hypodermic injection. All the experimental animals were monitored daily for signs of illness and deaths. LD_{50} (median lethal dose) was calculated by SPSS17 data analysis software.

Results and discussion

The *isrE* gene in *Salmonella enteritidis* wild type strain 50336 was cloned and DNA sequencing results suggested that the sequence of *isrE* in *Salmonella enteritidis* strain 50336 was the same as that in *S. typhimurium*. The deletion mutant 50336Δ*isrE* was constructed by the λ-Red-based recombination system to investigate the novel target genes and regulation function in virulence. DNA sequencing result showed that the mutant strain was constructed successfully, also identified by qRT-PCR. Then the target genes involved in virulence were screened by qRT-PCR. The results showed that mRNA levels of out membrane protein *ompD*, flagella subunit *fliC* and fimbriae subunits *fimA*, *sefA*, *csgA*, *csgD*, *sthA* were changed in the deletion mutant 50336Δ*isrE* compared to *S. enteritidis* 50336. When the deletion mutant 50336Δ*isrE* were grown to OD_{600} of 2.5 – 3.0, the decreased mRNA levels of *ompD*, *flic* and *fimA*, *sefA*, *csgA*, *csgD*, *sthA* were about 25, 8 and 3 – 5 folds respectively compared to the wild type strain. The ability of *S. enteritidis* 50336 and mutant strain 50336Δ*isrE* to adhere to Caco-2 cells was also investigated in this study. The deletion mutant strain showed a slight reduction in the ability to adhere to Caco-2 cells when compared to the wild type strain. Quantification of bacterial colony-forming units (CFUs) revealed that the *isrE* mutant exhibited about 30% less adherence than *S. enteritidis* 50336. Animal infections experiment was also performed to assess the virulence of *isrE* mutant strain. The LD_{50} of

the wild type strain 50336 and the mutant 50336Δ*isrE* were 2.809×10^8 cfu and 4.084×10^8 cfu respectively. It indicated that the deletion of *isrE* attenuated the virulence of *S. enteritidis* in chickens. In this study, we noticed that sRNA response quickly to the changing environment, the mRNA levels of *isrE* in different grown phase at OD_{600} of approximately 1.5, 2.0, 2.5 and 3.0 were detected by qRT-PCR. The result showed that the mRNA level of *IsrE* in wild type strain 50336 accumulated to peak at OD_{600} of 2.5 – 3.0, which was optimal as the phase for RNA extraction. Motility assays was also performed to identify the motility ability of the mutant strain 50336Δ*isrE*. The result showed that the mutant strain 50336Δ*isrE* exhibits less-mobile than the wild type strain which also confirmed the decrease of Flic. Another question is that the slight decreased adherence ability and virulence of the mutant strain 50336Δ*isrE* did not correspond to the significant change in mRNA levels of *ompD*, *fliC* and fimbriae subunits. The reason may be that the expression of some unknown target genes of IsrE involved in adherence ability and virulence was elevated in the mutant strain. The change of environment after bacteria added to cells may be another reason.

Conclusions

We cloned the *isrE* gene in the strain *S. enteritidis* 50336 and constructed the deletion mutant 50336Δ*isrE* successfully. Our study demonstrated that IsrE could decreased the mRNA levels of *ompD*, *flic* and fimbriae subunits *fimA*, *sefA*, *csgA*, *csgD*, *sthA*. The deletion of *isrE* could attenuate the adherence ability of *S. Enteritidis* to Caco-2 and its virulence in chickens.

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Virus-like Particles of Hepatitis B Virus Core Protein Containing Five Mimotopes of Infectious Bursal Disease Virus (IBDV) Protect Chickens Against IBDV

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Summary: Current infectious bursal disease virus (IBDV) vaccines suffer from maternal antibody interference and mimotope vaccines might be an alternative. Previously we demonstrated an IBDV VP2 five-mimotope polypeptide, 5EPIS, elicited protective immunity in chickens. In the current study, the *5epis* gene was inserted into a plasmid carrying human hepatitis B virus core protein (HBc) gene at its major immunodominant region site. The recombinant gene was efficiently expressed in *E. coli* to produce chimeric protein HBc-5EPIS which self-assembles to virus-like particles (VLP). Two-week old specific-pathogen-free chickens were immunized intramuscularly with HBc-5EPIS VLP or 5EPIS polypeptide without adjuvant (50 μg/injection) on day 0, 7, 14 and 21. Anti-5EPIS antibody was first detected on day 7 and day 21 in HBc-5EPIS and 5EPIS group, respectively; on day 28, anti-5EPIS titers reached 12800 or 1600 by ELISA, and 3200 or 800 by virus neutralization assay in HBc-5EPIS and 5EPIS group, respectively. No anti-5EPIS antibody was detected in the buffer control group throughout the experiment. Challenge on day 28 with a virulent IBDV strain resulted in 100%, 40.0% and 26.7% survival for chickens immunized with HBc-5EPIS, 5EPIS and buffer, respectively. These data suggest epitope presentation on chimeric VLP might be a promising approach for improving mimotope vaccines for IBDV.

Introduction

Infectious bursal disease virus (IBDV) causes severe immunosuppressive disease in young chickens. It continues to pose a significant threat to the poultry industry worldwide since its discovery over 40 years ago. IBDV is stable in the environment and can persist in poultry houses even after thorough cleaning and disinfection, is highly contagious and easily transmits through multiple routes and mediators; therefore, adequate control of IBD is only possible through vaccination [1].

Our group has previously tried a multi-mimotope vaccine approach to elicit anti-IBDV immunity, with an *E. coli* expressed polypeptide (termed 5EPIS) consisting of five mimotopes of VP2 protein, which were selected from a peptide phage-display library with anti-VP2 monoclonal antibodies and linked in tandem with short spacer sequence GGGS (Gly-Gly-Gly-Ser) [2]. Immunization of chickens with the five-mimotopes polypeptide 5EPIS in Freund's adjuvant conferred protection against challenge by a virulent IBDV strain. It is likely that the mimotope vaccine has the least interference from maternal antibodies, and is devoid of toxicities associated with administration of viral antigens. Thus it is a promising new strategy for developing recombinant subunit vaccine against IBDV. However, practical application of 5EPIS is limited by its small size (~9KDa) and thus weak immunogenicity. Approaches that improve the immunogenicity of mimotope polypeptides are therefore crucial to its field application.

One proven method of peptide antigen or epitope delivery is using virus-like particles (VLP), which, as inert, noninfectious, self-assembling particles, are effective immunogens due to their highly organized particulate structure [3]. Among these VLP immunogen carriers, Hepatitis B core (HBc) protein was the first reported and has remained one of the most promising delivery vehicles of foreign epitopes as it is highly immunogenic [4]. The high-resolution spatial structure of HBc icosahedrons shows that the major immunodominant region (MIR) is located on the tip of the spike of HBc particles, around the most protruding region between amino acids (aa) 78 and 82 [5]. For this reason, MIR is generally accepted as the target site of choice for insertion of foreign epitopes [4].

HBc-based VLP vaccine strategy has not been reported for IBDV. In this study, we made a chimeric protein by inserting nucleotide sequence encoding 5EPIS into a plasmid carrying the full-length HBc gene at the HBc MIR site and expressing the recombinant protein in *E. coli*, purified the protein, and determined whether HBc-5EPIS can self-assemble into VLP and whether fusion with HBc enhances 5EPIS-specific antibody response and protection against IBDV challenge.

Material and methods

The plasmids pET-HBc-*5epis* and pET-*5epis* were

separately constructed and transformed into *E. coli* BL21 (DE3) and the expression of the recombinant proteins were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 37℃. The bacteria were harvested and the purified HBc-5EPIS and 5EPIS preparations were respectively analyzed by SDS-PAGE and Western-blotting, and their purity was determined by densitometric scanning.

To obtain direct evidence of VLP formation, samples of the renaturized HBc-5EPIS and 5EPIS described above were respectively applied to a formvar-carbon 400 mesh coated copper grid and incubated for approximately 5 min. The samples were then viewed using transmission electron microscope.

To evaluate the efficacy of HBc as a carrier of 5EPIS to enhance 5EPIS-specific immune responses, 45 2-week-old specific-pathogen-free (SPF) White Leghorn chickens were randomly assigned to 3 groups of 15 birds each and placed into separate isolation houses. The immunization experiment was performed without any adjuvant. Each chicken in HBc-5EPIS group was injected intramuscularly with 50 μg purified HBc-5EPIS (in 100 μL) on day 0 and boosted on day 7, 14 and 21. In a second group, chickens were injected intramuscularly with 50 μg purified 5EPIS (in 100 μL) on day 0 and boosted on day 7, 14 and 21. Chickens in a third group were injected with PBS as negative control. Blood samples were taken from each bird prior to each injection and at one week after the last injection. Sera were prepared and used for detection of anti-5EPIS antibodies by indirect enzyme-linked immunosorbent assay (ELISA) in which the purified 5EPIS was used as coating antigen. The ability of the antibodies to neutralize the infectivity of the IBDV was determined using virus neutralization assay (VNA) in chicken embryo fibroblast cultures. Antibody titers were expressed as reciprocal of the highest serum dilution that showed positive reactivity in ELISA or neutralized 100 $TCID_{50}$ of IBDV-B87 strain in VNA.

All chickens in the HBc-5EPIS, 5EPIS, and PBS injected groups were challenged on day 28 with 200 ELD_{50} of a very virulent IBDV serotype I isolate GX8/99 in two of 0.1 ml, one by intranasal route and the second by intraocular route. The chickens were examined daily for clinical signs and mortality for 7 days post challenge.

Results and discussion

Both HBc-5EPIS and 5EPIS protein preparations were observed by negative staining electron microscopy for structure. Regular VLP with a diameter of around 45 nm was observed in the HBc-5EPIS but not the 5EPIS samples. The results confirmed that HBc kept its ability to form VLP with insertion of the five-mimotope polypeptide (5EPIS) of IBDV.

SPF chickens of 2 week old were immunized with HBc-5EPIS or 5EPIS protein intramuscularly for 4 times at one week interval at 50 μg protein per injection. In the HBc-5EPIS group, anti-5EPIS antibody was detectable as early as day 7 and the antibody level on day 28 reached to 12800 by ELISA or 3200 by VNA. In contrast, in the 5EPIS group, the anti-5EPIS antibodies became detectable on day 21 and the antibody titer on day 28 was only 1600 by ELISA and 800 by VNA. The PBS group showed no anti-5EPIS antibody during the whole experimental period, indicating no natural infection of IBDV occurred.

On day 28, the chickens were challenged with 200 ELD_{50} of a virulent IBDV strain GX8/99a. All chickens immunized with HBc-5EPIS (100%, 15/15) were protected. In addition, the chickens did not show any clinical signs or pathological lesions of IBDV infection. In contrast, chickens received 5EPIS or PBS injection succumbed to the challenge with survival rates of 40.0% (6/15) and 26.7% (4/15), respectively. The dead birds showed clinical symptoms, including inflammation, hemorrhage or atrophy in the bursa.

In this study, a chimeric VLP-forming protein HBc-5EPIS was generated by genetically inserting the IBDV five-mimotope polypeptide gene into the full-length HBc protein gene at the MIR site and expression by *E. coli*. The HBc-5EPIS protein was efficiently expressed in inclusion bodies, and after purification and renaturation, it self-assembled into VLP of ~45 nm in diameter and presented the 5EPIS domain on VLP surface. Immunization with HBc-5EPIS VLP with no adjuvants in chickens elicited significantly stronger anti-IBDV antibody responses and conferred greater protection against challenge by a virulent strain of IBDV than immunization with the 5EPIS polypeptide. These results confirmed that HBc-based VLP can be utilized as an epitope carrier for developing novel mimotope-based vaccines for IBDV, which might even be applied without using an adjuvant.

Conclusions

This is the first time chimeric HBc VLP displaying multi-mimotopes of IBDV is described. Our results clearly demonstrated that the potential usefulness of *E. coli*-derived HBc-based VLP as a carrier for immunogenic presentation and delivery of multi-mimotopes 5EPIS and the possibility of using HBc-5EPIS as a new type of IBD vaccine. In addition, this study also suggests a prospective future for HBc particles to be utilized as useful tools for exploring multivalent or combined vaccines for the prevention and control of avian infectious diseases.

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Expression of CYP1A1, CYP2E1 and CYP3A1 in Sudan Ⅰ—Induced Hepatic Injury

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Summary: To disscuss the effect of sudan Ⅰ expressions of CYP1A1, CYP2E1 and CYP3A1 in Rat Liver, the experiment is the detection of enzymatic activity, half-quantitative analysis of RT-PCR. Experimental and real-time PCR examination. Animal were randomly divided into two group: control group, sudan Ⅰ group, The sudan Ⅰ group was divided into four does group. Liver and blood were collected for test 10 days after treatment. These results indicated that CYP1A1, CYP2E1 and CYP3A1 gene were increase in a certain dose range, compared with control group, the activity of CYP2E1, CYP3A1 in Rat Liver Microsome were increase in a certain dose range, compared with control group. This result point out that sudan Ⅰ impacted the activity and expression of CYP1A1, CYP2E1 and CYP3A1, educed various kinds poisoning effects, leaded to the poisoning of organism and injury of Rat Liver.

Introduction

Sudan red dye is a group of synthetic lipophilic azo compound, which is common used as industrial dyes. But some people commonly add sudan red dye in food. Sudan Ⅰ is classified as the third class of carcinogens in International Agency for Research on Cancer, it has the risk of potentially carcinogenic [1, 2]. In 2005, "McDonald's fast-food chain" events which contained sudan Ⅰ cause great concern of many domestic and foreign scholars. CYP450 enzyme system is supergene family which composed a group of many isozymes, including the CYP1A subfamily, CYP2E subfamily and CYP3A subfamily, papers have shown that when sudan red dye et al xenobiotics entered into the body, it will enhance the activity of the enzyme and introduce hepatic injury if the enzymes involved in the metabolism of the substance in the body [3, 4]. Although people have a certain recognize of the pathogenic mechanism of sudan red dye in recent years, Sudan red dye on the pathogenic mechanism of hepatic injury only restrictes to CYP1A1, CYP2E1 and CYP3A1 have not been reported.

The experiment from the point of view on integrative medicine establishes animal models of liver pathological injury, and provided the experimental basis for recognize sudan red dye-induced hepatic injury mechanism and poisoning pathologic evaluation criteria.

Material and methods

We use the following reagents: sudan Ⅰ, the reagent of Trizol, DEPC, RT-PCR kit and so on. Experimental animal are randomly divided into two group: control group, sudan Ⅰ group, the sudan Ⅰ group was divided into four does group. Liver and blood were collected for test 10 days after treatment. The liver tissue collection: after killing the rats, livers of 150 mg are loaded in sterile vials and immediately placed in liquid nitrogen. Preparation of liver microsomes is the method of calcium precipitation.

The experiment is CYP2E1, CYP3A1 activity of the detection by spectrophotometry, the expression quantity of CYP1A1, CYP2E1 and CYP3A1 by half-quantitative analysis of RT-PCR, CYP1A1 gene copynumber by real-time PCR examination.

Results and discussion

The experimental results show that: sudan Ⅰ treatment group was significantly higher than control group ($P < 0.05$, $P < 0.01$), and in a certain dose range, the expression of CYP1A1, CYP2E1 and CYP3A1 gene is rendered as an upward trend. But CYP1A1, CYP3A1 gene in the IV group show a slight decrease, CYP2E1 geneperform decreasing in group Ⅲ (Table 1, 2, Fig. 1). CYP1A1 genecan analyse results by real-time quantitative RT-PCR are consistent with the results of semi-quantitative analysis, it shows that sudan Ⅰ treatment group was significantly higher than control group ($P < 0.05$, $P < 0.01$), and it shows an upward trend in a certain dose range. Thus it can be seen that in the process of metabolism of exogenous poisons, CYP1A1, CYP2E1 and CYP3A1 are in the important position.

Table 1 CYP1A1, CYP2E1, CYP3A1 gene expression in Liver

Groups	CYP1A1/CYC	CYP2E1/CYC	CYP3A1/CYC
C	1.23 ± 0.16	1.04 ± 0.11	1.08 ± 0.13
Ⅰ	1.71 ± 0.18*	1.16 ± 0.16*	1.29 ± 0.11*
Ⅱ	2.03 ± 0.22**	1.55 ± 0.13**	1.31 ± 0.09**
Ⅲ	2.20 ± 0.24**	1.49 ± 0.16**	1.41 ± 0.10**
Ⅳ	2.08 ± 0.25**	1.31 ± 0.12**	1.40 ± 0.15**

Table 2 CYP1A1 gene copynumber in Liver

Groups	CYP1A1 expression
C	0.00049 ± 0.0021
Ⅰ	0.00299 ± 0.0013 *
Ⅱ	0.01225 ± 0.0014**
Ⅲ	0.03441 ± 0.0028**
Ⅳ	0.02701 ± 0.0019**

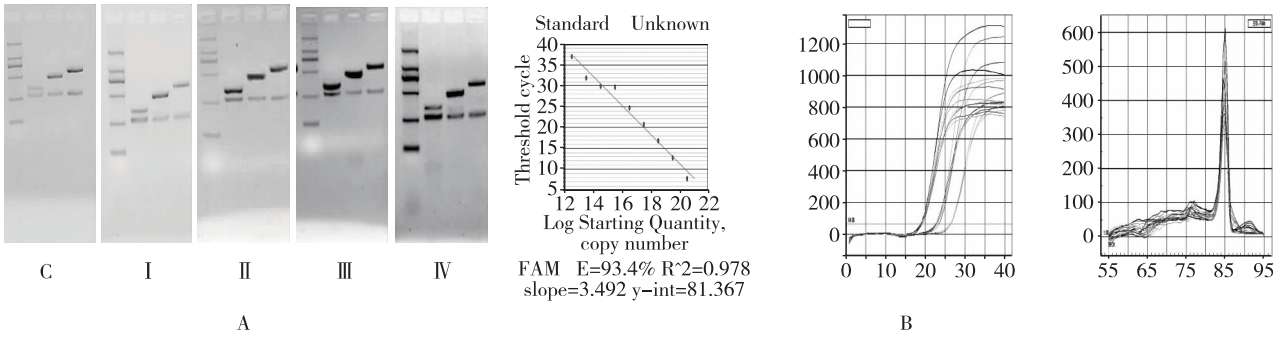

Fig. 1 The results of RT-PCR target productsamd the real time PCR figure
A. From left to rightrespectively: Control group, group Ⅰ, group Ⅱ, group Ⅲ, group Ⅳ (M. 2000 marker; 1. Upper strap for CYP1A1, subtus strap for CYC; 2. Upper strap for CYP2E1, subtus strap for CYC; 3. Upper strap for CYP3A1, Upper strap for CYC.); B. The real time PCR standard curve of CYP1A1, dynamic curve of CYP1A1 and melt curve of CYP1A1

Through the research of 30 years, molecular mechanisms of sudan red dye toxicity have not fully investigated, but there is a certain understanding of its pathogenic mechanism. The studies show that metabolite of sudan red dye with liver DNA form adducts and introduce DNA oxidative damage, they play a part in related gene expression by inducing CYP450 enzyme system, changing enzyme activity, inducing generation of DNA adduct and reducing the functional liver cells.

Studies confirmed that CYP1A1 plays a role in many cancer pathogenesis, therefore CYP1A1 expression level is considered to be the potential indicators of chemical carcinogenesis [5, 6]. Expression levels of CYPlA1 activity are higher after inducing in certain inducers such as dioxins, the induction mechanism is realated to aryl hydrocarbon receptor, aryl hydrocarbon receptor can increase the activity of CYP1A1 by inducing the expression of the CYP1A1 gene to hepatic injury[3,4]. After environmental toxicants such as polycyclic aromatic hydrocarbons, polychlorinated dibenzo-dioxins, polychlorinated biphenyls enter into the body or be joined the cells cultured in vitro, they can be formed prone cyclization reaction under the catalysis of CYP1A1 and generate epoxy compounds and they can attack macromolecules such as proteins, nucleic acids and lead to hepatocytes toxic effects[7]. Some exogenous poisons can be metabolism through CYP2E1 and can induce their levels elevated of CYP2E1 gene expression. Exogenous poisons metabolize to liver toxicity of the active intermediate products, which start membrane lipid peroxide, lead to change of intracellular and extracellular Ca^{2+} concentration equilibrium, destruct the body's important antioxidant glutathione and cause hepatocytes damage[8]. Xenobiotics will start the biotransformation after entering into the body, biotransformation mainly catalyzes by CYP 450. CYP3A1 is the most important metabolic enzyme gene in the CYP450 gene family, this type of enzyme is significance in many aspects of toxicology and drug metabolism[9,10].

In the whole sudan Ⅰ control group, rat liver microsomal CYP2E1 and CYP3A1 show to increase enzyme activity and to be ascendant trend in a certain dose range. The result shows that sudan Ⅰ can affect hepatic CYP450 enzyme system, that the enhancements of CYP2E1 and CYP3A1 activity affect the normal function of the hepatocyte and lead to damage of the liver of animal organism(Table 3).

Table 3 Activity of CYP2E1, CYP3A1 in Rat Liver Microsome

Groups	CYP2E1 activity	CYP3A1 activity(nmol/g·min)
C	132.17 ± 20.46	24.83 ± 7.06
Ⅰ	164.5 ± 11.89*	47.33 ± 8.33*
Ⅱ	211.67 ± 25.47**	50.17 ± 13.99*
Ⅲ	209.00 ± 26.67**	70.33 ± 12.82**
Ⅳ	203.67 ± 16.51**	68.83 ± 8.31**

If body occur poison, they can generate toxic products through metabolism of hepatic CYP450 enzyme system, such as electrophilic base, free radicals, oxygen free radicals, toxic products can cause biological

membrane lipid peroxide damage by the function of mitochondria, nuclear[11 – 13]. CYP450 enzyme system are able to metabolize exogenous compounds, which often engender activity and increase content.

Conclusions

This study shows that sudan Ⅰ may promote the expression of liver CYP1A1, CYP2E1 and CYP3A1 gene, this is an important molecular mechanisms on sudan Ⅰ-introdued liver damage in metabolism. Enzyme activity of elevated CYP2E1 and CYP3A1 indicate that it play an important role in the process of sudan Ⅰ-induced hepatic injury.

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The Effect of Sudan Ⅰ on Exressions of CYP 2E1

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Summary: The protein and mRNA of CYP 2E1 are detected by Immunohistochemistry (IHC) and in situ hybridization (ISH). The experiment is to observe the effect of Sudan Ⅰ on expression of CYP 2E1 and hepatic injury. Experimental animals are randomly divided into control group (C group) and Sudan Ⅰ treatment group. The results of IHC examination show that IOD of CYP 2E1 protein has increase ($P < 0.01$) in livers in Sudan Ⅰ treatment groups and show uptrend in certain dose range. The results of ISH examination show that IOD of CYP 2E1mRNA is significant increase ($P < 0.01$) in livers in Sudan Ⅰ treatment groups and show uptrend in certain dose range. The results indicate that Sudan Ⅰ can induce increase of the expressions of CYP 2E1.

Introduction

Sudan Ⅰ is a kind of lipophilicity azo compounds, and is used as chemical stain[1]. Studies show that liver is the main target organ of Sudan Ⅰ, which induces pathological damage of liver and genotoxicity[2,3].

At present, the toxic mechanisms of histopathology on Sudan Ⅰ are rarely reported. The experiments will build the poisioning animal model of Sudan Ⅰ to discuss the effect of Sudan Ⅰ on liver of mouse by integrative medicine. This study uses immunohistochemistry (IHC) and in situ hybridization (ISH) to detect the expressions of CYP 2E1 in mouse livers and provide the mechanisms of toxicity and pathogenesis on Sudan Ⅰ.

Material and methods

Main reagents: Sudan Ⅰ (Sinopharm Chemical Reagent); CYP 1A1 PcAb (ShangHaiJingTian Biotechincco); CYP 2E1 PcAb, SABC-POD Kit, DAB Kit (WuHan Boster Bio-Engineering Limited company).

Mouse: SD mouses (Kunming Medical University), 140 – 160 g, are rondomly divided into control group (C group, $n = 6 \times 1$) and Sudan Ⅰ group ($n = 6 \times 4$). Feedstuff is mixed with Sudan Ⅰ and compounded in four dose groups: Ⅰ group (265 mg/kg)、Ⅱ group (530 mg/kg)、Ⅲ group (795 mg/kg) and Ⅳ group (975 mg/kg). Blank group is fed with normal feedstuff. The feeding is lasted for ten days.

Sample preparation: The mouse liver was fixed in 4% paraformaldehyde PBS that was disposed by DEPC. When the liver is collected, we must be careful of RNase pollution. The hepatic tissues are used for ISH. Others are fixed in 10% neutral formalin and are used for IHC.

Methods: Immunohistochemistry is used to detect the expression of CYP 2E1 by SABC (Strept Avidin-Biotin Complex)[4 – 6]. In situ hybridization is used to detect the expression of CYP 2E1 mRNA by the probe marked with digoxin. Experimental data are analysed by SPSS. The diversity between groups is analysed by *t*-test.

Results and discussion

IHC of CYP 2E1 protein in liver

Fig. 1 and Fig. 2 are the results of IHC.

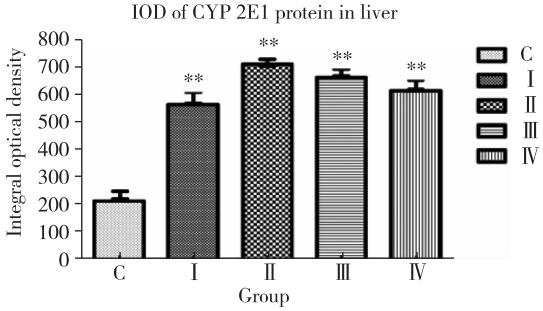

Fig. 1 IOD of CYP 2E1 protein in liver (n = 6). The IOD of Group Ⅰ, Ⅱ, Ⅲ and Ⅳ have increase in certain dose range and is significantly more than Control group ($P < 0.01$).

ISH of CYP 2E1 mRNA in liver

Fig. 3 and Fig. 4 are the resutls of IHC.

CYP 2E1 can catelyze toxic precursor to intermediate products with hepatotoxicity And the final toxicity to liver is directly related to the activity of CYP 2E1[7,8]. The results of the experiment show that Sudan Ⅰ can induce the increased expression of the gene of CYP 2E1 and can happen not only in expression level of RNA, but also in protein level.

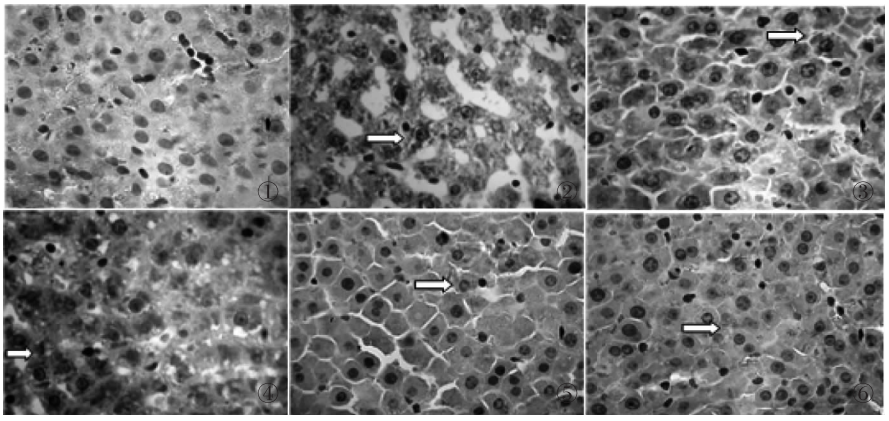

Fig. 2 The expression of CYP 2E1 protein in liver(400 ×). The expression of CYP 2E1 protein of Group Ⅰ, Ⅱ, Ⅲ, Ⅳ is more than Control group in cytoplasm. ① Negative control: No brown positive materials; ② Control group: Positive materials are found in cytoplasm; ③ Group Ⅰ: More positive materials are found, compared with Control group; ④ Group Ⅱ: Positive materials rapidly increase, compared with Group Ⅰ; ⑤ Group Ⅲ: Positive materials decrease, compared with Group Ⅱ, but are more than Control group; ⑥ Group Ⅳ: Positive materials decrease, compared with Group Ⅲ in cytoplasm, but are more than Control group.

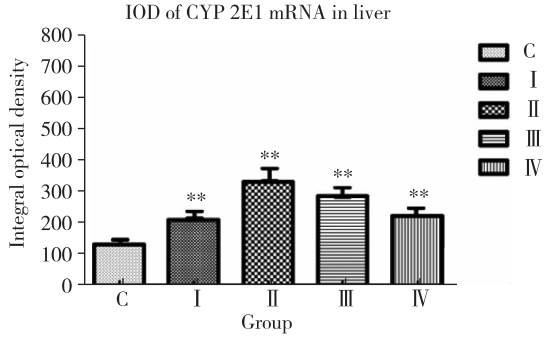

Fig. 3 IOD of CYP 2E1 mRNA in liver(n = 6). The IOD of Group Ⅰ, Ⅱ, Ⅲ and Ⅳ have increase in certain dose range, and is significantly more than Control group($P < 0.01$).

This study showed the result that Sudan Ⅰ could increase the expression of CYP 2E1 was same with the reported experimental results [9 – 11]. Because the activity of CYP 2E1 has the positive correlation with the level of the toxicity, the increase of the expression of CYP 2E1 may induce the addition of toxic products in Sudan Ⅰ metabolism of the body and can also promote the increase of the toxicity to liver.

Conclusions

Sudan Ⅰ increases the expression of CYP 2E1 in mouse liver and promotes Sudan Ⅰ metabolism into toxic substances. The result is one of the important mechanisms which Sudan Ⅰ induces the liver injury.

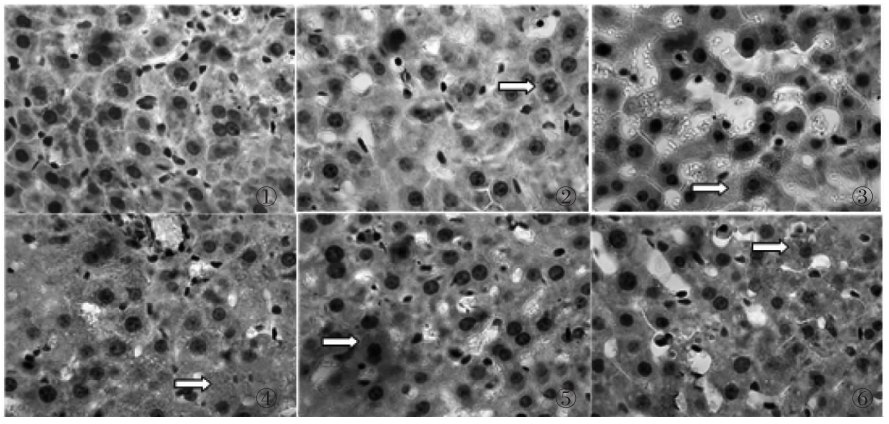

Fig. 4 The expression of CYP 2E1 mRNA in liver(400 ×). The expression of CYP 2E1 mRNA of Group Ⅰ, Ⅱ, Ⅲ, Ⅳ is more than Control group in cytoplasm. ① Negative control: No brown positive materials; ② Control group: Positive materials are found in hepatocyte; ③ Group Ⅰ: More positive materials are found, compared with Control group; ④ Group Ⅱ: Positive materials obviously increase, compared with Group Ⅰ; ⑤ Group Ⅲ: Positive materials decrease, compared with Group Ⅱ, but are more than Control group; ⑥ Group Ⅳ: Positive materials decrease, compared with Group Ⅲ in cytoplasm, but are more than Control group.

Acknowledgment

This study was supported by the Program for Innovative Research Team (in Science and Technology) in University of Yunnan Province, the Technology system Construction of swine industry in modern agriculture of Yunnan Province.

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Analysis of the Effect of Copper on the Virulence of a Pathogenic *Escherichia coli* Strain

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Summary: To investigate the effect of copper on the pathogenic STEC, *Escherichia coli* (*E. coli*) strain 107/86 cultured in the medium complemented with 1.0 mmol/L copper was examined for any potential alteration for virulence. The results showed that Cu-treated strain 107/86 displays suppressed growth profile, an impaired ability to adhere to IPEC-J2 cells, and decreased Stx2e production. Reduction of adherence ability under copper treatment was confirmed by QRT-PCR. Meanwhile, the overall bacterial virulence was also attenuated. The Cu-treated 107/86 strain differed from the untreated strain with 33-fold lower activity of AI-2 in quorum sensing system. Less biofilm formation and decreased diameters of flagella motility halo were also detected. Cu-treated strain exhibited 6-fold higher production of outer membrane protein. The medium with copper has multiple adverse effects on 107/86 resulting in significant attenuation of bacterial virulence. This study provides new insights to the antimicrobial effect of copper.
Key words: *Escherichia coli*, copper, virulence

Introduction

Both porcine edema disease (ED) and post-weaning diarrhea (PWD) caused by Shiga toxin-producing *Escherichia coli* (STEC) result in important morbidity and mortality, and are economically important diseases in pig industries. F18 fimbriae confer to STEC the ability to attach to receptors on the enterocytes, and mediates the colonization in the small intestine. The virulence factor Shiga toxin (Stx) has its cytotoxicity to Vero cells. Besides fimbriae and Stx toxin, recent studies have showed a number of other factors that are involved in the development and maintenance of bacterial virulence. For example, AI-2 mediated quorum-sensing system which likely participates in bacterial virulence by regulating the production of virulence factors such as biofilm and flagellum.

As a potentially toxic heavy metal, copper has long been known to be effective against pathogenic organisms that are associated with plant and animal diseases, that is why copper has been used extensively in agriculture and animal farming industries as a pesticide. For instance, copper has been used in shrimp farming as a management procedure for disease control. Although the exact mechanisms of the antimicrobial property in copper are still unclear, previous studies have shown that copper can reduce the growth of bacterial pathogens like *Staphylococcus aureus*, *Edwardsiella tarda* and impair the virulence of viruses like influenza virus and human immunodeficiency virus.

In this study, we analyzed the copper effect on the virulence of a pathogenic STEC strain and found that copper stress exerts a widely negative effect on the virulence of 107/86 strain.

Material and methods

Bacterial strain and growth conditions

E. coli 107/86 (wild type, O139: H1: F18ab, Stx2e) was routinely cultured in Luria broth (LB) plates at 28 ℃ and 37 ℃, respectively. For Cu treatment, 107/86 was grown in LB medium containing 1.0 mmol/L $CuSO_4$ to late-logarithmic phase and transferred to fresh LB medium containing 1.0 mmol/L $CuSO_4$. The cells were transferred into LB medium without copper, used for the assays.

MIC of copper in STEC 107/86 strain

A volume of 100 μL of 107/86 strain was plated on LB plates supplemented with $CuSO_4$ with the concentration from 1.0 mmol/L to 6.0 mmol/L. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of $CuSO_4$ that prevented growth.

Growth curve

Cu-treated and untreated bacteria were cultured, cultures were taken at various time points for measurement of OD_{600}.

Bacterial adherence assays

Fimbriae-mediated binding ability of STEC 107/86 strain was determined by a quantitative adhesion assay previously described (Scaletsky Silva & Trabulsi, 1984).

Quantification of bacterial number in mouse feces

Four-week-old ICR mice into three groups were used

in this study. The Cu-treated and Cu-untreated 107/86 strains were injected intragastrically into mice at 2×10^8 CFU concentration, while the control was injected with sterile PBS. Fecal pellets were collected every day after infection and homogenized in 5 mL of PBS. 10-fold dilution series were plated on MacConkey agar plates to determine the number of CFU of *E. coli* (van der Velden, Baumler, Tsolis, & Heffron, 1998).

Bioluminescence detection of AI-2

The AI-2 bioassay was performed to assess the activity of quorum sensing system in bacteria according to the protocol previously described (Han & Lu, 2009).

Motility assay

1 μL of Cu-treated and untreated 107/86 strains were used to seed in the middle of motility plate containing 1% tryptone, 0.25% NaCl, and 0.3% agar (Sperandio, Torres & Kaper, 2002). The motility halos were measured to evaluate motility.

Quantification of biofilm formation

10 μL of Cu-treated and untreated 107/86 were seeded into biofilm inducing medium(Li et al., 2008) in glass test tubes or 96-well plate. OD_{600} values of each well were read to measure the amount of adherent crystal violet.

Extraction of outer membrane protein

Cu-treated and untreated bacteria were suspended in PBS with 5% Triton X-114. After incubation for 8 h at 4℃, cellular debris was removed. The supernatant was warmed at 37℃ for 15 min for phase separation. After centrifugation for 15 min at 5,000 *g*, the upper aqueous phase was separated from the detergent phase. The procedure was repeated twice to obtain the cleaned-up detergent phase. Ethanol was added into detergent phase for 8h incubation at −20℃. Next, the solutions were centrifuged at 10,000 *g* for 30 min to get the OMP extractions (Tibor, Decelle & Letesson, 1999). BCA Kit was performed to quantitatively determine the amount of OMP from Cu-treated and untreated strains.

Cytotoxicity of Stx2e toxin to Vero cell

Briefly, Cu-treated and untreated strains were cultured to a density of 0.3 measured in OD_{600}, then mitomycin C was added into each tube to the final concentration of 0.25 μg/mL(de Sablet et al., 2008). After 12 h induction, supernatants of the strains cultures were filtered through a 0.22 μm filter. 100 μL of supernatants containing toxin was added to the Vero cells and incubated for 20 h. Crystal violet solution was added and stained the remain cell after wash. Ethanol was added to resolubilize the adherent crystal violet (Akiyoshi et al., 2005).

Cytotoxicity of total bacteria to Vero cell

Briefly, cells were rinsed twice and 10^8 CFU strains were added for 3 h incubation. The wells were then washed, fixed and stained by crystal violet. Ethanol was added to measure OD_{600} value.

RNA isolation and Real-time RT-PCR

Total RNA from Cu-treated and untreated strains were extracted using TRNzol method respectively (Duan et al., 2012). Primers designed to amplify *fimH*, *fedF*, *fliC* were targeted to regions of unique sequence within each gene, which play significant roles in construction of type I fimbriae, F18 fimbriae, and flagellum. Assays were performed in triplicate with the ABI 7500 (Applied Biosystems, Foster City, CA, USA). All data were normalized to the endogenous reference gene gapA. The data were analyzed by the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All statistical analyses were performed using SPSS 15.0 software. Differences in data were analyzed with *t*-test.

Results and discussion

Effect of copper treatment on growth profiles of 107/86 strain

MIC assay showed that for STEC 107/86, the MIC of $CuSO_4$ is 5.2 mmol/L. Therefore 1.0 mmol/L copper concentration was determined to influence bacteria in this study. Growth profiles showed that compared to untreated 107/86, Cu-treated strain grew slower and reached a lower maximum cell density when cultured in medium containing 1.0 mmol/L copper.

Effect of copper on adhesion of 107/86

Cu-treated 107/86 showed nearly 40% reduction in adherence to IPEC-J2 cells when compared to the untreated strain. Bacteria were recovered from the feces pellets on days 1, 2, 3, 4 and 5 postinfection to monitor intestinal colonization and adhesion ability of STEC 107/86. Increased numbers of Cu-treated bacteria were recovered from fecal pellets on day 1 an day 2 postinfection. Meanwhile, data from bacteria recovered after day 3 postinfection indicates that there are very small differences between Cu-treated and untreated strains.

Effect of copper on quorum sensing system

Culture supernatants from positive control BB120, STEC 107/86 and Cu-treated 107/86 strain induced an increase of 73-fold, 69-fold and 2.2-fold, respectively, compared with the negative control.

Effect of copper on biofilm formation

107/86 strain has been tested as having ability to form biofilm, whereas 4.2-fold decreased biofilm formation was observed when it was cultured in the copper-complemented medium.

Effect of copper on motility

Motility halo of the strain under copper stress was smaller. The diameters of untreated strain were nearly

130% larger.

Copper decrease cytotoxicity of Stx2e and total bacteria

In both cytotoxicity assays from Stx2e toxin and total bacteria, cell damage caused by Cu-treated strain were decreased by 33% or 25%.

Effect of copper on outer membrane protein production

Phase partitioning method was performed by Triton X-114 to extract OMP. OMP from Cu-treated 107/86 was nearly 6-fold of OMP from untreated strain.

Transcriptional analysis of effect of copper

The results pointed that copper treatment can cause a 3.5 fold decrease in expression of *fliC*, 6.2 fold decrease in *fedF*, while nearly 3.8 fold decrease can be found in mRNA level of *fimH*.

Growth profiles showed that Cu-treated 107/86 grew slower and can only reached a much lower OD_{600} value in stationary phase of bacteria growth, which exhibits that 1.0 mmol/L copper in medium impaired the growth of this strain. The most studied virulence factors of STEC are adhesins, toxins and cytotoxins. Here we showed that copper can influence negatively the expression of F18 fimbriae, are involved in the adherence to IPEC-J2 cells, and are important in adherence to intestinal epithelial cells in mouse model. Cu-treated 107/86 exhibited magnitude reduction in adherence to cells. Meanwhile, on the first day after infection, more number of Cu-treated strain were detected in feces, which demonstrated that untreated 107/86 have higher adherence to mouse intestinal. QRT-PCR method was also exerted to test expression level of adhesion-associated genes like F18 fimbriae gene *fedF*, flagellum gene *fliC*, and type I fimbriae gene *fimH*. The 3 to 6 folds decrease of the mRNA level of the genes confirmed the results from adhesion experiments that adherence of strain was attenuated by copper treatment. After adhesion to host cells, virulence factor shiga toxins inhibit protein synthesis by cleaving a specific adenine residue in the 28S subunit of eukaryotic rRNA. They are cytotoxic for Vero tissue culture cells. To assess the Stx2e production, extracted Stx2e and total bacteria were added into wells covered by Vero cells. In both assays, more survival cells were detected by crystal violet stain method after invasion of Cu-treated strain, which suggests that the production of Stx2e in 107/86 strain was reduced under excessive copper concentration in environment.

To many living organisms, copper ion is a kind of essential trace metal which acts as a catalyst or cofactor in many enzymes. However, copper is also highly toxic to cells, as it is redox active and thus can generate reactive oxygen species. As a result, bacteria have developed various strategies to maintain their copper homoeostasis. Growth of 107/86 in medium containing copper led to significant increase of OMP production. Although the mechanism employed by STEC to deal with copper concentration is not thoroughly clear, it is possible that OMP perform an important role in maintain the balance of copper in organisms, therefore the production of OMP of 107/86 strain undertook a powerful induction from the environment with high copper concentration.

Bacteria use a chemical language of molecules in a process called quorum sensing (QS), which adjust bacterial behavior in response to environment. QS implies that bacteria sense each other and the population by detecting a threshold accumulation of signals called auto-inducers, which exert important role like regulating genes. Many studies have highlighted the significance of AI-2 in the biological processes of various bacteria including biofilm formation, motility and virulence expression. Some studies have shown that in many bacteria like *Edwardsiella tarda*, *Vibrio harvey*, *E. coli*, *Yersinia enterocolitica*, the AI-2 activity was influenced by excessive copper stress, which was also proved in this study. Based on bioluminescence assay, a 50-fold lower activity of AI-2 was detected. Previous studies had found the links between quorum sensing and biofilm formation and motility. Biofilm is reported to be affected by AI-2 in a wide range of bacteria. It is obvious that the Cu-treated strain formed biofilms much weakly. In many cases, biofilm is essential virulence factor in contributing to colonization, immune escape and antibiotics resistance. Extended motility halos were detected in motility assays of untreated strain. Although the role of flagella in *E. coli* pathogenesis has not been sufficiently explored,, flagella was involved in bacterial adhesion, motility, potent stimuli of innate immunity. We assumed that through quorum sensing, many properties of pathogenesis have been adjusted under copper stress.

Conclusions

In conclusion, this study demonstrates that copper in medium affects the expression of important virulence factors. These results suggest that given the negative effects on *E. coli* growth and pathogenesis, copper was proved to be an option to control bacterial virulence and may be applied as an antimicrobial agent.

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Monomeric Flagellin May be More Suitable as an Adjuvant Than Polymeric Flagellin

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Summary: This paper describes the biological differences between monomeric and polymeric flagellin. Bacterial flagellin, an effective immune adjuvant, can be expressed as a monomer or polymer depending on the folding and assembly process. However, the biological differences between monomeric and polymeric flagellin are unclear. Here, we prepared the monomer (mfliC/M) and polymer chimeric flagellin (pfliC/M) containing the ectodomain of the matrix 2 protein of influenza A virus in the hypervariable region of fliC flagellin to compare their biological efficacy. The results indicated that they showed distinct circular dichroism spectra and that the polymerization of mfliC/M was much lower than for pfliC/M. Three hours after transfection into macrophages, mfliC/M could induce more higher interleukin-1β secretion. Serum antibody assay indicated that pfliC/M could induce significantly higher flagellin-specific antibody production two weeks after immunization compared with mfliC/M. Thus, the folding and assembly processes of flagellin might contribute to their difference and monomeric flagellin may be more suitable as an adjuvant than polymeric flagellin.

Introduction

Bacterial flagellum is the motility device of bacterium and its elongated filament is composed primarily of many subunits of flagellin [1]. The N-and C-terminal regions of *Salmonella typhimurium* phase 1 flagellin, encoded by the *fli*C[i] gene, have been reported to be highly conserved helix structure across different bacterial species to maintain the integrity of the filament structure [2]. Whereas the middle regions are hypervariable regions (HVR). During bacterial flagellar filament assembly, flagellin has to be exported from the cytoplasm to the tip of the growing filament and undergo a process from unfolding to folding state [3,4]. Because the folding and assembly process is essential for flagellin to be expressed on the bacterial surface [5], the recombinant flagellin can be acquired as unfolding form within the cytoplasm (monomer) [6,7] or folding form showing on the surface of bacteria (polymer) [8].

Bacterial flagellin is a potent trigger of host innate immune responses in eukaryotes [9]. Two forms of flagellin have been found to show different ascendency in the immune response against exogenous antigen, although they have all been reported the adjuvanticity [10,11]. Besides, the residues 89 – 96 of flagellin which are involved in Toll-like receptor (TLR)-5 signaling is hidden in the flagellum and only monomer is accessible for recognition by TLR-5 [12]. Hence the folding and assembly process of flagellin may influence their biological function. It is reported that flagella combined with exogenous antigen can significantly promote the release of cytokine, when compared with flagella alone [10]. Thus, for the flagellin-specfic response assay, the present of exogenous antigen is more practical for the vaccine development than flagellin alone.

Replacement of part of the HVR of flagellin by exogenous antigen is a potentially effective strategy for the vaccine development [13]. The monomeric flagellin expressed in *E. coli* and the polymeric form directly isolated from *Salmonella* are usually used in the vaccine development [11, 14]. Reports have shown that the linkage of flagellin with the ectodomain of matrix 2 protein of influenza A virus (M2e) is a good vaccine candidate [14]. In order to assay the biological activities of two forms of flagellin, two tandem M2e genes were designed to replace the DNA sequence (607 – 684 base pairs) of the *fli*C[i] gene in HVR and the chimeric genes were expressed in *E. coli* and *Salmonella* to produce the monomer and polymer chimeric flagellin, respectively. Then, the biological characteristics including protein conformation, polymerization, IL-1β release and antibody production in the early stage of immunization were analyzed.

Material and methods

Mice and bacteria

Six-week-old female C3H/HeJ and C57BL/6 mice were obtained from the Comparative Medicine Center of Yangzhou University (Yangzhou, China). This study was carried out in accordance with the regulations set

forward by the Chinese Ministry of Science and Technology. The protocol were approved by the Committee on the Ethics of Animal Experiments of Yangzhou University (Permit Number: 2007-0005). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Recombinant plasmid pET-*fli*C containing *fli*C^{i} gene of *S. typhimurium* LT2 in the *Bam*H I/*Xho* I sites of pET30a + was constructed by Dr. Hui Zhang [13]. *E. coli* BL21(DE3), *S. typhimurium* LB5000 and aflagellar *S. dublin* SL5928 were used in the following cloning procedures. The wild-type flagellin of *S. typhimurium* SL7207 was used as a control.

Preparation of monomer and polymer chimeric flagellin

The DNA fragment (5'-GATACTACGATTGCTTTAGACAATAGTACTTCCCTGCTGACCGAAGTTGAAACCCCGACCCGTAACGAATGGGAATGCCGTTGCTCCGATTCCTCCGATGGCGGCGGCTGCTCCCTGCTGACCGAAGTTGAAACCCCGACCCGTAACGAATGGGAATGCCGTTGCTCCGATTCCTCCGATAAATATTACGCCAAAGTTACCGTTACGGGG-3') was synthesized by GenScript Company (Nanjing, China). In this sequence, two M2e gene (4 – 72 bp) were connected with a DNA sequence, 5'-GGCGGCGGCTGC-3', to yield the M2e2 gene (underlined). At the two ends of the M2e2 gene, a partial HVR sequence of *fli*C^{i} gene was included for overlap polymerase chain reaction (PCR) procedures. The 5'-terminal region of the *fli*C^{i} gene, *fli*C-AC, was amplified from pET-*fli*C plasmid using the following primers: fliC-forward-A (5'-AGGGATCCATGGCACAAGTCATTAA-3') and fliC-reverse-C (5'-AGTACTATTGTCTAAAGCAATCG-3'). The 3'-terminal region, *fli*C-DB, was amplified using the following primers: fliC-forward-D (5'-AAATATTACGCCAAAGTTACCG-3') and fliC-reverse-B (5'-TGCTCGAGTTAACGCAGTAAAGAGA-3'). The *fli*C-AC-M2e2 fragment was amplified using the following primers: fliC-forward-A and M2E2-reverse (5'-CCCCGTAACGGTAACTTTGGCG-3') from *fli*C-AC and M2e2. The chimeric *fli*C/M2e2 gene was amplified using the following primers: fliC-forward-A and fliC-reverse-B from *fli*C-AC-M2e2 and *fli*C-DB. The PCR product was cloned into the *Bam*H I/*Xho* I sites of pET30a + to yield the recombinant plasmid, pET-*fli*C/M2e2.

For the expression of monomer chimeric flagellin, plasmid pET-*fli*C/M2e2 was transformed into BL21 (DE3). The recombinant bacteria, BL21 (DE3) (pET-*fli*C/M2e2), were cultured in LB agar plates containing 50 μg/ml kanamycin overnight at 37℃ and the kanamycin-resistant colonies were then amplified by culturing with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The expression of 6 × His-tagged unfolding chimeric flagellin (mfliC/M) was identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis using a murine anti-flagellin polyclonal antibody. The recombinant bacteria BL21 (DE3) (pET) was used as a negative control. Affinity purification of His-tagged mfliC/M was performed with a Nickel bead column (Qiagen, Valencia, CA) according to the manufacturer's instructions after supersonic schizolysis of the recombinant bacteria.

For the expression of polymer chimeric flagellin, plasmid pET-*fli*C/M2e2 was electroporated into LB5000 and then transferred to SL5928 by P22HT*int* transduction, as previously described [13]. The growth of chimeric flagellin, pfliC/M, in the surface of kanamycin-resistant SL5928 (*fli*C/M2e2) was observed by Tecnai-12 transmission electron microscopy (Philips, Holland) after negative staining. After subculture, genomic DNA and plasmids of SL5928(*fli*C/M2e2) were extracted and PCR amplification of *fli*C/M2e2 using the primers fliC-forward-A and fliC-reverse-B and M2e2 gene using the primers M2E2-forward (5'-GATACTACGATTGCTTTAGACAATA-3') and M2E2-reverse were performed to identify whether the *fli*C/M2e2 gene was integrated into the chromosome. Polymeric pfliC/M was extracted by acid treatment followed by ammonium sulfate precipitation, as previously described [11].

The purity was identified by SDS-PAGE and the morphology of bacteria and purified proteins were observed by Tecnai-12 transmission electronic microscopy (Philips, Holland).

Circular dichroism

To investigate the secondary structure of purified flagellin, pfliC/M and mfliC/M, the far-ultraviolet (UV) circular dichroism (CD) spectra were recorded using a J-810 spectropolarimeter (JASCO, Tokyo, Japan) using 0.2 cm pathlength cuvettes at 20℃. The concentration of all samples used was 50 μg/ml in 10 mM PBS, pH 7.4. The CD spectra of PBS was used as baseline. The mean spectra of three repeated scans in the range between 190 and 250 nm, corrected by subtraction of the buffer signal, were obtained by taking points every 0.1 nm with 1 nm slit width.

Dynamic light scattering

To further investigate the polymerization of mfliC/M in solution, the dynamic light scattering (DLS) measurements for pfliC/M and mfliC/M were performed with a EEN 3690 Malvern Zetasizer (Malvern, Worcestershire, UK) at 20℃. The concentration of all samples used was adjusted to 50 μg/ml in PBS (pH 7.4).

IL-1β release

Primary peritoneal exudate cells (PECs) of C57BL/6 mice were harvested by washing the peritoneal cavity with RPMI 1640 medium containing 10% fetal bovine

serum (1640-FBS, Gibco, Carlsbad, CA), as previously described (Kumari and Saxena, 2011). Cells were plated in 96-well plates at 2×10^4 cells/well and incubated for 3 h at 37℃ with 1 μg/ml *E. coli* lipopolysaccharide (LPS). The supernatants were removed and the cells were softly washed with 1640-FBS. pfliC/M and mfliC/M were then transfected respectively using lipofectamine 2000 (Invitrogen, Carlsbad, CA) and incubated for 3 h (Simon and Samuel, 2008). The non-transfected cells were used as controls. The secretion of IL-1β in supernatant was detected using a mouse enzyme-linked immunosorbent assay (ELISA) set (Biosciences, PharMingen, San Diego, CA).

ELISA

C3H/HeJ mice were subcutaneously injected with a single dose of purified wild-type flagellin, pfliC/M and mfliC/M (10 μg per mouse), respectively. Mice subcutaneously injected with PBS were used as controls. Serum samples were collected by orbital vein bleeding at one-week intervals from 1 to 3 weeks after immunization. The flagellin-specific antibody levels were detected by indirect ELISA. Briefly, 96-well microplates were coated with purified wild-type flagellin (1 μg/well) overnight at 4℃. The plates were blocked, washed and then incubated with two-fold serial dilution of serum samples for 2 h at 37℃. Diluted (1∶3000) anti-mouse IgG conjugated with peroxidase (Zymed, San Francisco, CA) was then added and incubated for 2 h at 37℃. The reacting solutions were read at OD_{405} using a microplate reader of Anthos 2010 (Anthos Labtec Instruments GmbH, Wals, Salzburg, Austria).

Statistical analysis

Within each experiment, three to four replicate assays were conducted for each treatment. All statistical analyses were performed by Student's *t*-test using SPSS software (Version 13.0 for Windows, Chicago, IL). A value of $P \leqslant 0.05$ was considered to be significant.

Results and discussion

In this study, the chimeric gene *fli*C/M2e2 was designed to express for monomeric and polymeric flagellin, respectively. mfliC/M was successfully expressed in recombinant bacteria BL21(DE3)(pET-*fli*C/M2e2) when compared to BL21(DE3)(pET). In the pET-*fli*C/M2e2 plasmid, the plasmid sequence containing a 6 His-tag coding sequence was designed to fusion express with *fli*C/M2e2 gene for easy purification of mfliC/M. After subculture of SL5928 (*fli*C/M2e2), flagella were observed on the bacterial surface. The *fli*C/M2e2 gene and M2e2 gene could be amplified by PCR from the genomic DNA of SL5928 (*fli*C/M2e2), but not from plasmids or SL5928 genomic DNA. This indicated that the *fli*C/M2e2 gene was integrated in the chromosome of SL5928 through homologous recombination. SDS-PAGE results indicated that the purified pfliC/M and mfliC/M were approximately 50 and 60 kDa, respectively. Besides the post-translational modification, one reason for the differences between the two flagellin forms may be due to the expression of the plasmid sequence (150 bp) in mfliC/M. By transmission electron microscopy, polymeric pfliC/M showed a filament shape similar to wild-type flagellin and was significantly different from monomeric mfliC/M.

CD spectra is a common method for the analysis of protein structures in solution [15]. CD spectra between 190 and 250 nm are used to analyze the secondary structure of protein. In this study, significantly different CD spectra at this region were found between monomeric mfliC/M and polymeric pfliC/M. The CD signal of pfliC/M was stronger than that of mfliC/M. Similar CD spectra were observed between pfliC/M and wild-type polymeric flagellin of *S. typhimurium* SL7207. The polymerization of proteins in solution can be determined by the size distribution by volume. In this study, the mean dynamic radii of pfliC/M measured by DLS were larger than that of mfliC/M, though a few polymers were present in the mfliC/M solution. This indicated that the polymerization degree of pfliC/M was higher than that of mfliC/M. In the filament assembly of bacterial flagellum, the unfolding subunit protein flagellin has to be transported via a narrow channel, and then fold before being assembled into the growing filament [3, 4]. The transport process is accompanied with the change of protein comformation. In the study, the same chimeric gene was expressed as monomeric or polymeric flagellin. The folding and assembly of polymeric flagellin is one of the major difference compared with monomeric flagellin. The significant difference of CD spectra and polymerization between mfliC/M and pfliC/M may be due to this process. The terminal regions of flagellin are conserved helix structure to maintain the integrity of the filament structure [2]. The CD spectra of pfliC/M in this study confirmed this point. Whereas only low-level polymerization of mfliC/M were found *in vitro*, so we suppose that the polymerization of pfliC/M *in vivo* is a complex process. The middle region of flagellin is a HVR varying greatly in size and composition in different strains, and the insertion of exogenous antigen into HVR does not interfere with the assembly and export function [2,3]. This study also verified that replacement of part of the HVR by M2e did not interfere with the conformation of polymeric flagellin.

Flagellin can activate NLRC4 inflammasome to induce the maturation and secretion of IL-1β [16]. LPS does not activate NLRC4 [17], but can induce the production of pro-IL-1β [18]. In this study, three hours after transfection, pfliC/M and mfliC/M could induce the

secretion of IL-1β in PECs compared with the untransfected group ($P < 0.05$). The secretion level of IL-1β trggered by pfliC/M was lower than that by mfliC/M ($P < 0.05$). The C-terminal amino acid sequence of flagellin can be detected by NLRC4 in macrophages and then trigger the release of IL-1β [19]. In *in vitro* experiments, the secretion of IL-1β can be stimulated after transfection of monomeric flagellin to PECs [18]. In the study, both polymeric and monomeric flagellin could induce the secretion of IL-1β. The relatively higher levels of IL-1β secretion induced by mfliC/M compared with pfliC/M may be due to the direct recognition of monomeric flagellin by NLRC4 in the early stage, whereas polymeric flagellin must dissociate to release the monomeric form of flagellin first.

LPS is an agonist of TLR-4 signaling (Lahiri et al., 2008). As C3H/HeJ mice are TLR-4 mutated, they were selected for the immune assay to exclude the interference of LPS. The sera antibody titers specific for flagellin were detected by indirect ELISA, using plates coated with wild-type flagellin (1 μg/well). No significant difference was found between mfliC/M-immunization and the control group, but a greater antibody response was induced by pfliC/M compared with mfliC/M ($P < 0.05$) two weeks after subcutaneous immunization. Three weeks later, the antibody titers induced by pfliC/M increased and were at similar levels as those induced by wild-type flagellin, while antibody titers were still significantly lower in the mfliC/M-immunization group ($P < 0.05$). For the immunogenicity assay, mice were given a dose of 10 μg flagellin per mouse, which is pathophysiologically and clinically relevant [20]. Interestingly, in the early stage of immunization, pfliC/M could induce strong flagellin-specific antibody production, but not for mfliC/M. Polymeric flagellin has been reported to induce strong antibody response [21,22]. The folding and assembly process was not happened in mfliC/M. Associated with the difference in CD spectra between mfliC/M and pfliC/M, it may be suggested that the conformational epitope of flagellin produced by the folding and assembly process is a immunodominance epitope.

Conclusions

Two forms of flagellin produced like these have been used as adjuvant in the vaccine development. Monomeric flagellin may be more suitable as an adjuvant than polymeric flagellin. The different biological activities found in this study will be benefit for their use in the future.

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The Role of Hsp90α in Apoptosis and Damages of Primary Myocardial Cell Cultures of Neonatal Rats after Heat Stress

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Summary: This study describes the relation among the heat induced pathological changes and apoptosis, and the variations of protective Hsp90α and its corresponding mRNA in the heat stressed primary myocardial cells of neonatal rats *in vitro*. Levels of enzyme CK increased from the beginning of heat stress and acute cellular lesions characterized by vacuolar degeneration and necrosis in the heat stressed cells were observed after 40 min of heat stress, suggesting that the myocardial cells *in vitro* were obviously damaged by higher temperature. The levels of cleaved Caspase-3 and Cytochrome C which concerned to apoptosis increased significantly after 40 min of heat stress while Hsp90α protein significantly decreased, and the levels of cleaved Caspase-3 and Cytochrome C decreased while Hsp90α significantly increased after 6 h of heat stress, indicating that early depletion of Hsp90α coincides with a high rate of necrosis and apoptosis in heat stressed myocardial cells while in surviving cells Hsp90α increases after 6 h of heat stress again with significantly less apoptosis.

Introduction

High stress such as transport stressed pigs and heat stressed chicken cause eventually heat shock and sudden death in a number of these animals[1]. The underlying mechanisms leading to cellular damage and death of animals stressed in such a way are not yet well understood although a number of factors and physiological reactions are already known since long[2]. Apoptosis is one of biochemical events which can occur in all tissues including heart cells where it causes a loss of adult cardiomyocytes[3] Hsps, are highly conserved proteins whose expression in cells of warm blooded animals[4], are induced in response to a wide variety of physiological and environmental influences, including heat exposure [5]. Hsp90α, a main type of HSP90 acts as a good mediator of cellular stress[6]. However, the mechanisms responsible for the protective function of Hsp90α are not fully understood. Therefore, the purpose of this study was to understand the relation among the heat stress induced pathological changes and apoptosis of heat stressed myocardial cells, and the variations of protective Hsp90α and its corresponding mRNA *in vitro*.

Material and methods

Primary myocardial cells of neonatal rat were incubated at 37℃ in humidified atmosphere of 5% CO_2 and 95% air for 72 h. The cells were heat stressed *in vitro* for 10 min, 20 min, 40 min, 1 h, 2 h, 4 h, 6 h and 8 h respectively in another incubator with 95% air and 5% CO_2 at 42℃. The control group was kept at 37℃. The myocardial cells grown on polylysine-coated coverslips were fixed with 4% for cytopathological examination. The myocardial cells used to detect *hsp*90α mRNA, Hsp90α protein expression, Caspase-3 and Cytochrome C. All analysis were performed using a one way analysis of variance (ANOVA) using the Statistical Package for Social Sciences (SPSS version 16.0). Differences were regarded as significant at $P < 0.05$.

Results

CK increased gradually from the beginning of heat stress and reached the highest level at 2 h of heat stress ($P < 0.05$) (Fig. 1). Vacuolar and granular degeneration and even necrosis after 40 min and 2 h of heat stress (Fig. 2). Levels of Caspase-3 (Fig. 3) and Cytochrome increased significantly at 40 min of heat stress compared to control and Hsp90α expression levels

in the myocardial cells significantly decreased after 40 min of heat stress (Fig. 4).

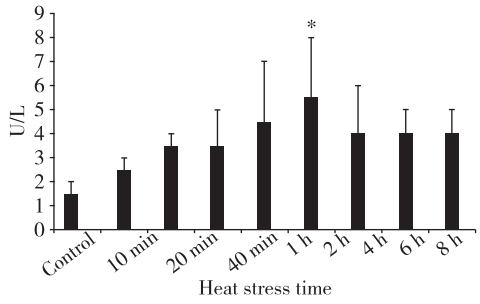

Fig. 1 Levels of enzyme CK in the heat stressed myocardial cells of rat (U/L)

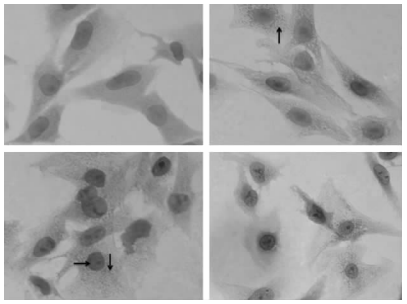

Fig. 2 Representative photomicrographs of primary myocardial cells

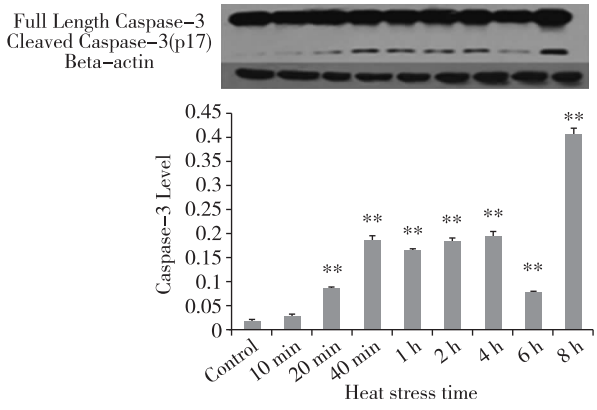

Fig. 3 Levels of Caspase-3 in the heat stressed myocardial cells

Discussion

The CK increment gradually from the beginning of heat stress are in line with the cytopathological changes of the heat stressed myocardial cells characterized by vacuolar and granular degeneration, and even necrosis after 40 min and 2 h of heat stress. It indicates that heat stress induce myocardial cell damage as earlier as heat treatment. A similar result demonstrated that heat stress

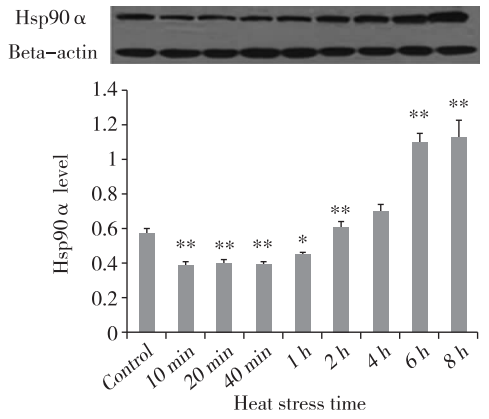

Fig. 4 Levels of Hsp90α in the heat stressed myocardial

cause myocardial cell damage in broilers *in vivo* which accompanied by an elevation of the enzyme creatine kinase (CK) and an increase in apoptotic cells in consistent with our results[7]. Levels of Caspase-3 and Cytochrome C which concerned to apoptosis increased significantly at 40 min of heat stress, indicating that the heat induced myocardial cell apoptosis is increased from early period of heat shock. The lower concentration of Hsp90α expression in the early phase of heat stress could be used as a risk indicator of pathological damage and massive apoptosis possibly followed by heart failure. However the results also displayed that Hsp90α was elevated at 6 and 8 h of heat stress, while the levels of Caspase-3 and Cytochrome C decreased at 6 h of heat stress, meanwhile the levels of enzyme CK decreased, indicating that the damages of the heat stressed myocardial cells begin to reduce after 6 and 8 h of heat stress. These imply that the apoptosis of the myocardial cells happened through the activation of the Cytochrome C and Caspase-3 pathway. However, the cell repair capacity of Hsp90α is overstrained in the early phase of heat treatment and needs some hours to stabilise. Hsps can inhibit or aid the apoptotic mechanism through their chaperone functions by affecting protein folding, ubiquitin degradation pathways and protein translocation[8].

Conclusions

Heat stress induces cellular damage and apoptosis of myocardial cells during the first stage of heat treatment. Low Hsp90α indicates that the repair capacity of this protein is overstrained in the first phase of sudden heat stress in myocardial cells and needs some hours to stabilise. Hsp90α have protective function against damage at the end period of the heat exposure.

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Investigation of the Expression and Subcelluar Locolization of αB-Crystallin and HSF-1 in Rat Primary Myocardial Cells Subjected to Heat Stress

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Summary: The aim of this study is to investigate the role of αB-crystallin and Heat shock factor-1 (HSF-1) in cell protection under heat stress at 42℃. According to our experiment, it appears that HSF-1 stimulated an increase in αB-crystallin transcription following exposure to heat stress in rat myocardial cells. However, the levels of the αB-crystallin protein did not correlate with the increase in mRNA levels, suggesting discordance between transcription and translation. These findings may suggest that the consumption of αB-crystallin exceeded its production in myocardial cells after a short duration of heat stress. Sufficient levels of αB-crystallin to maintain normal cellular functions were restored after the heat stress had ceased.

Introduction

Stresses such as heat, animal transportation, and chemical factors contribute to lethal pathological symptoms related to cardiovascular disease, such as cardiac arrhythmias, seizures, or hypovolemic shock with tachycardia and eventual circulatory collapse [1].

Heat shock proteins (HSP) are ubiquitously expressed and highly conserved in prokaryotes and eukaryotes[2]. The HSP are reported to have roles in protein intracellular transport, cytoskeletal architecture, mutation masking, regulation of translation, intracellular redox homeostasis and protection against spontaneous or induced programmed cell death[3]. αB-crystallin is a member of the family of small heat shock proteins[4], which displays chaperone-like properties, including the ability to prevent the accumulation of denatured proteins and increase cellular tolerance to stress[5]. However, the mechanisms responsible for the protective functions of αB-crystallin are not fully understood. In the absence of cellular stress, Heat shock factor-1(HSF-1) is repressed via its association with Hsp. However, in response to stress, HSF-1 binds to specific sequences in the promoters of Hsp and stimulates their expression[6].

The mechanisms by which stress causes cell damage and alterations in cellular metabolism are difficult to investigate systematically in vivo due to the numerous confounding environmental variables. Therefore, rat myocardial cells were subjected to heat stress in vitro, as a model system to examine the correlation between variations in the levels of αB-crystallin and HSF-1 and cellular damage. Of particular interest was the role of HSF-1 in regulating the transcription of αB-crystallin after heat stress, and the potential role of αB-crystallin in protecting against hyperthermia-induced cellular damage.

Material and methods

Cell culture and heat treatment

Neonatal rat primary myocardial cells subjected to heat stress were placed at 42℃, whereas the control cells remained at 37℃. One plate from each group was removed from the incubator at the start of the experiment (0 min) and after 10, 20, 40, 60, 120, 240, 360, and 480 min.

Enzymatic activities of heat-stressed myocardial cells

Myocardial cell supernatants were collected from the heat stress and control groups, and the activities of lactate dehydrogenase (LDH), aspartate aminotransferase (AST), creatine kinase (CK) and creatine kinase MB (CK-MB) were measured according to the instructions included in the commercial kits (Nanjing Jiancheng Biochemical Reagent Co., Nanjing, China).

Immunofluorescence

Myocardial cells were grown on glass coverslips and fixed in 4% paraformaldehyde for 30 min at room temperature (RT) and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS). After blocking with 5% skimmed milk in PBS for 1 h, an anti-rat αB-crystallin monoclonal antibody (1∶500; ab13496, Abcam, USA) or an anti-rat HSF-1 monoclonal antibody (1∶100; ab61382, Abcam, USA) were added to the coverslip, and incubated in a moist chamber for 1 h at

37℃. A rhodamine red-conjugated goat anti-mouse IgG antibody (1∶100; Boster, China) was added and then incubated at 37 C for 1 h. Furthermore, the coverslips were stained with DAPI solution. Finally, the coverslips were dry-mounted and observed under the fluorescence microscope (Cx41-32rfl, Olympus, Japan).

Isolation of total RNA and RT-PCR

Total RNA was isolated from myocardial cells in the heat stress and control groups using TRIzol reagent, according to the manufacturer's instructions (Invitrogen, USA). 2 μg of each sample was reverse-transcribed using the Transcript M-MLV kit (Invitrogen, USA) following the manufacturer's protocol and stored at −80℃.

Primer design

The primer sequences were as follows:

αB-crystallin sense: 5′-GCACGAAGAGCGCCAG-GACGA-3′

αB-crystallin antisense: 5′-CGTCGGCTGGGATCC-GGTACT-3′

hsf-1 sense: 5′-ACCCCAGCCTCTGCCTGCT-3′

hsf-1 antisense: 5′-TTCCCACTCGGGCTCCAGCA-3′

β-actin sense: 5′-CCCATCTATGAGGGTTCA-3′

β-actin antisense: 5′-TCACGCACGATTTCC-3′

The expected lengths of the PCR products for αB-crystallin, *hsf-1* and β-actin were 134 bp, 153 bp and 128 bp, respectively.

Quantitative real-time polymerase chain reaction (QPCR)

The thermal profile was established according to the manufacturer's protocol. Briefly, this protocol consisted of enzyme activation at 95℃ for 3 min, followed by 45 cycles of denaturation at 95℃ for 5 s and annealing and elongation at 52℃ for 30 s. A negative control without DNA was included in each experiment. A 2-fold dilution series of the template was used in the QPCR reactions. The αB-crystallin and *hsf-1* mRNA expression levels in each sample were normalized using the following formula: Relative quantity of αB-crystallin/*hsf-1* mRNA = $2^{-\Delta\Delta Ct}$

ΔΔCt = − [(CtαB-crystallin/*hsf-1* mRNA − Ctβ-actin mRNA) control group − (CtαB-crystallin/*hsf-1* mRNA − Ct β-actin mRNA) test group]

Semi-quantitative detection of αB-crystallin and HSF-1 protein levels in primary myocardial cells

Proteins were extracted from heat-stressed myocardial cells and boiled for 5 min, 10 μg proteins were loaded on 10% SDS-PAGE gels for electrophoresis, then transferred onto a nitrocellulose membrane by electrotransfer. Membrane was blocking with 5% skimmed milk for 1 h at RT. The membranes were incubated with anti-rat αB-crystallin monoclonal antibody (1∶1000; ab13496), anti-rat HSF-1 monoclonal antibody (1∶1000; ab61382) or anti-rat β-actin monoclonal antibody (1∶1000; ab8224; all from Abcam, USA) for 16 h at 4℃. The membranes were incubated with peroxidase-conjugated goat anti-mouse IgG antibody at RT for 1 h, finally, the antibody-antigen complexes were detected using Western blotting luminal reagent. The intensity of each band was normalized to the intensity of β-actin protein.

Results and discussion

Enzymatic activities in cellular supernatants

The activity of several enzymes related to myocardial cell damage, AST, LDH, CK and CK-MB, was assessed in the supernatants of the myocardial cells, as shown in Fig. 1.

The activity of the CK and CK-MB enzymes was significantly higher in the supernatant of heat-stressed myocardial cells compared with control myocardial cells ($P < 0.01$). AST level was increased as early as 10 min following the transfer of cells to the higher temperature. These data suggest that myocardial cells were already showing signs of damage after only 10 min of heat stress at 42℃, and also that the level of AST is useful as an early indicator of cardiac damage.

Subcellular localization of αB-crystallin and HSF-1 in myocardial cells

The subcellular localization of αB-crystallin and HSF-1 proteins in the neonatal rat primary myocardial cells was determined by immunocytochemistry (Fig. 2 and 3, respectively). the intensity of the granular, cytoplasmic αB-crystallin staining by immunofluorescence clearly decreased after 60 min of heat stress, but was then strongly induced after 240 min of heat stress. suggesting that the degradation of αB-crystallin exceeded its production at that time. A steady-state level of αB-crystallin protein appears sufficient to maintain normal cellular function and viability during heat stress.

HSF-1 was stably and consistently located within the nucleus of myocardial cells in vitro before and during heat stress. Therefore, it appears unlikely that HSF-1 translocation plays any role in the multistep process of HSF-1 activation that has been described in other cell types.

αB-crystallin, *hsf-1* mRNA and protein levels in heat-stressed myocardial cells *in vitro*

The expression levels of αB-crystallin mRNA and protein were examined in rat myocardial cells, and normalized to β-actin mRNA or protein levels (Fig. 4).

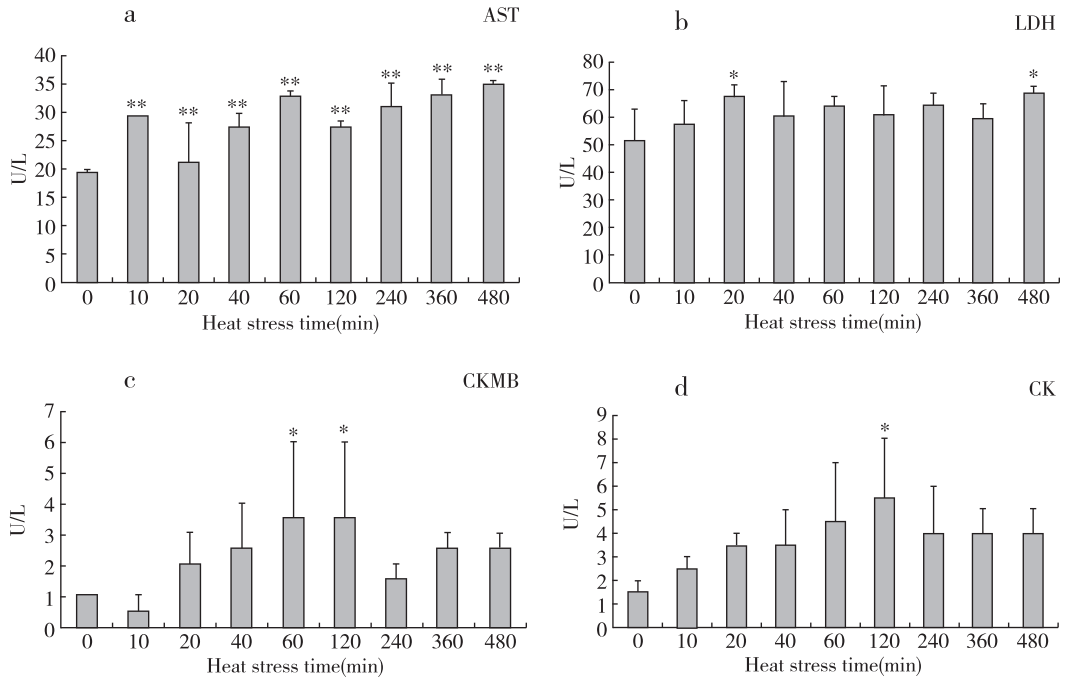

Fig. 1 The activity of key cellular damage enzymes in the supernatant of myocardial cells following heat stress
$^{*}P<0.01$; $^{**}P<0.05$

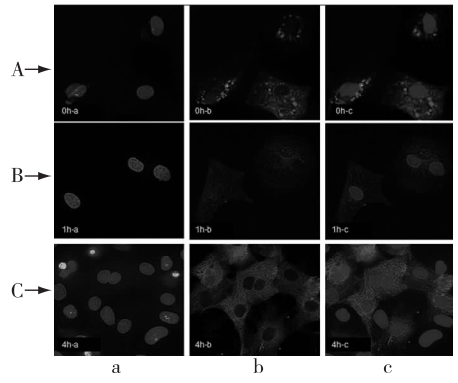

Fig. 2 Subcellular localization of αB-crystallin in primary rat myocardial cells after heat stress(40 ×)
a: DAPI (nuclei); b: αB-crystallin (rhodamine); c: merge. A: 0 h at 37℃; B: 1 h heat at 42℃; C: 4 h at 42℃

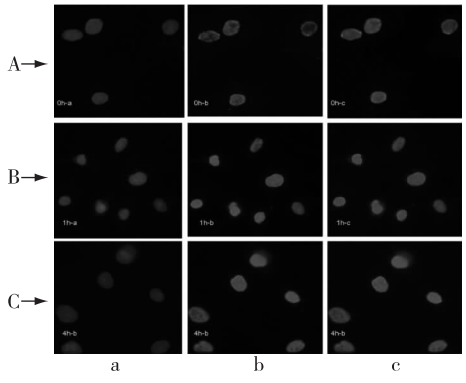

Fig. 3 Subcellular distribution of HSF-1 in primary rat myocardial cells after heat stress(40 ×)
a: DAPI (nuclei); b: αB-crystallin (rhodamine); c: merge. A: 0 h at 37℃; B: 1 h heat at 42℃; C: 4 h at 42℃

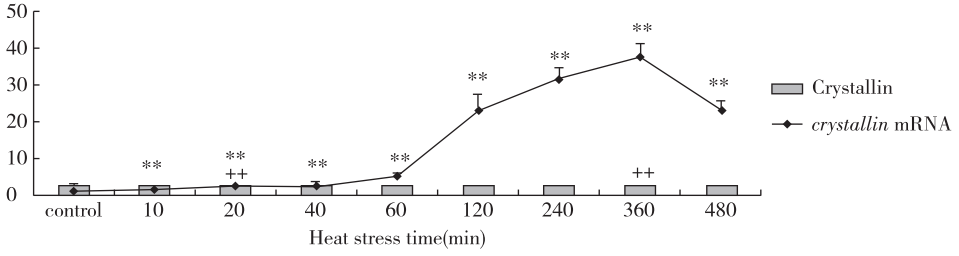

Fig. 4 Levels of αB-crystallin mRNA and protein in heat-stressed myocardial cells
***αB-crystallin* mRNA: $^{*}P<0.05$, $^{**}P<0.01$; αB-crystallin protein: $^{++}P<0.01$**

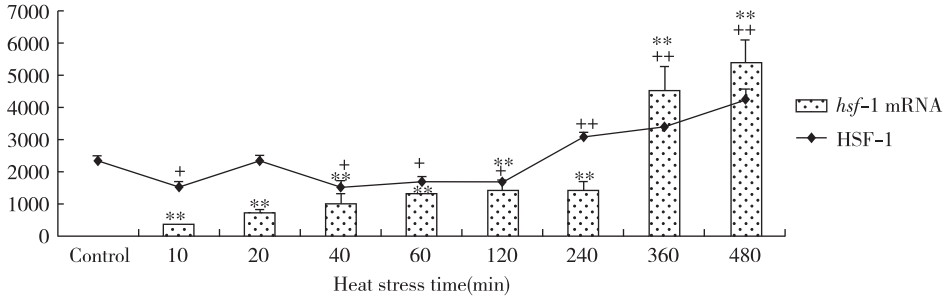

Fig. 5 Levels of HSF-1 mRNA and protein in heat-stressed myocardial cells
***hsf-1* mRNA: $^{*}P<0.05$, $^{**}P<0.01$; HSF-1 protein: $^{++}P<0.01$**

Western blotting analysis indicated that the levels of αB-crystallin remained fairly constant throughout the time-course, with the exception of statistically significant increases at 20 min and 480 min of heat stress ($P<0.01$). In contrast, αB-crystallin mRNA levels increased rapidly, within 10 min of the cells being subjected to heat stress, and reached maximal levels after 360 min of heat stress. Therefore, the mRNA and protein levels of αB-crystallin were not correlated in the myocardial cells of neonatal rats after heat stress. The rate of synthesis of the αB-crystallin protein appeared to be decreased at the early stages of heat stress in vitro, suggesting that the degradation of αB-crystallin exceeded its production at that time.

The expression of HSF-1 mRNA and protein was examined in rat myocardial cells, and normalized to β-actin *mRNA or* protein levels (Fig. 5). The levels of *hsf-1* mRNA increased rapidly and in a step-wise manner throughout the time-course, and reached maximal levels (3-fold induction) after 360 min of heat stress. HSF-1 protein levels initially decreased in the myocardial cells exposed to heat stress, and these lower levels were sustained until 240 min of heat stress. After that time, significant increases in HSF-1 protein were observed from 360 min until the end of the experiment ($P<0.01$). We observed fluctuations in the expression of HSF-1 mRNA and protein within the first 120 min of heat stress, suggesting that HSF-1 was required for additional pathways, in addition to the induction of αB-crystallin gene expression[7]. Alternatively, HSF-1 may become depleted at the early stages of heat stress as the cells begin to adapt to new environmental conditions.

Conclusions

In our study, heat-stressed rat primary myocardial cells model was established. αB-Crystallin which belongs to small heat shock protein can protect cells from being damage due to hyperthermia. Heat shock factor-1 has a role in regulating the transcription of *αB-Crystallin* mRNA during heat stress.

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Morphological Supervision of the Sugar Beet Depending on Ways of Processing of the Soil of the Territory of Northern Prikaspy

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In article phenological supervision of a sugar beet are considered. Development of a root crop of a sugar beet in the first year of life. The tsvetushnost reason-early crops in cold long spring and long light day is mentioned. Researches were carried out in 2008 for 2012 to the GNU Near-Caspian research institute of arid agriculture.

Research work on studying and selection of new highly productive grades for a semidesertic zone was carried out with group of hybrids of a sugar beet Lgovsky MS-29 and Ramonsky MS-46.

The purpose of the real work was studying and selection of highly productive hybrids of a sugar beet on богаре and in the conditions of an irrigation at various processing of the soil for Northern Prikaspy's territory. For improvement of cleaning of root crops of a sugar beet it is necessary to investigate in a complex their technical characteristics, i. e. morphological properties. Obobshchenna results for years of researches of hybrids of a sugar beet on development phases, on soil processings, and also at various humidity of the soil.

Thus, as a result of the carried-out researches at the main processing of the soil phenological phases proceeded in both options more long, than at direct crops. So, at otvalny processing at an irrigation harvesting of root crops at a hybrid Lgovsky MC-29 began on September 10, and at direct crops on September 08. As a result of long finding of root crops of a sugar beet in the soil, in them collect sugar, thus when ripe roots reached physiological maturity root. thereby when maturing root crops physiological ripeness of a root crop is reached.

At germination of seeds, at first the germinal back and a subcotyledonous knee start in growth. Two cotyledons at an exit to a surface turn green and carry out functions of leaves (phase "forks"). Through 6 – 8 days after shoots are formed 1 couple real leaves, behind it there are 2 – 3 couples. At this stage органогенеза there is a change of anatomic structures, or a root molt. Further leaves are developed already on one. In the beginning they appear through everyone 2 – 3 days, and in the middle of vegetation -through 1 – 2 days. At the end of vegetation emergence of leaves is slowed down. In the first year of life of a plant of beet form 60 – 90 leaves which 70 days remain active during 60. Leaves of an average circle (from 10 to 25) are most productive. Duration of vigorous activity of each leaf about 25 days. By the time of cleaning net productivity of photosynthesis decreases, the mass of leaves decreases. Optimalnaya Square of leaves on 1га a beet plantation makes— 40 – 50 thousand sq. m [2].

In the first year of life of sugar beet it is possible to allocate three periods. During the first period of a plant vigorously form leaves and root system, root crop growth in thickness lags behind growth of leaves (May to June). During the second period the strengthened growth of a root crop and leaves (July to August) is observed. For the third period it is characteristic the slowed-down gain of leaves and intensive accumulation of solid (September to October).

In the first year of life of a plant on a root crop head in a bosom of each leaf sleeping kidneys for which development lowered temperatures— 0 – 8℃ are necessary are stuffed up. The top kidneys formed in the fall, develop under more favorable conditions. High-quality changes for transition to flowering and fructification at kidneys come to an end in the fall or in the spring of the next year, after disembarkation of root crops are formed цветоносы on which flowers and seeds [1] are formed.

Sometimes at part of plants of sugar beet deviations from a normal biennial cycle of development -from crops of seeds before harvesting of seeds are observed. In this case at separate plants the full cycle of development of sleeping kidneys and formation of tsvetonosny escapes occur in the first year of life, this phenomenon is called as a tsvetushnost. The tsvetushnost reason-early crops in cold long spring and long light day. Tsvetushnye root crops low-sugary and rough, at storage are surprised kagatny decay more strongly.

Some of the root crops landed for the second year for the seed purposes, on the contrary, don't give tsvetonosny escapes and continue to form only the socket of leaves.

Such plants are called as "pigheads". They appear under the influence of increased temperatures during early cleaning, owing to autumn and spring drying of the uterine root crops, the increased temperature at storage. "Pigheads" start fructifying for the third year. Presence of "pigheads" among vysadkov-seed plants considerably reduces a crop of seeds [3].

Researches were conducted in 2008 – 2012 in the GNU Caspian research institute of arid agriculture.

Research work on studying and selection of new highly productive grades for a semidesertic zone was carried out with group of hybrids of sugar beet Lgovsky MS-29 and Ramonsky MS-46.

The purpose of the real work was studying and selection of highly productive hybrids of sugar beet on борape and in the conditions of an irrigation at various processing of the soil for Northern Prikaspy's territory.

For improvement of cleaning of root crops of sugar beet it is necessary to investigate in a complex their technical characteristics, i. e. morphological properties. [2]. Data of our researches testify that the phenological condition depends on ways of processing of the soil.

Thus, at the main processing of the soil phenological phases proceeded in both options more long, than at direct crops. So, at dump processing at an irrigation harvesting of root crops at a hybrid Lgovsky MC-29 began on September 10, and at direct crops on September 08. As a result of long finding of root crops of sugar beet in the soil, in them collect sugar, thereby when maturing root crops physiological ripeness of a root crop is reached.

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Variations of Hsp60 and HSF-1 in Primary Myocardial Cells of Rats Under Various Durations of *in vitro* Heat Stress

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Summary: The relationship between the heat stress induced variations of protective Hsp60 and its regulating factor HSF-1 expression in the heat-stressed primary myocardial cells of neonatal rats in *in vitro* was studied by using enzyme detections, immunoblotting and qPCR. The enzymes elevations displayed injuries of myocardial cells after heat exposure at 42℃. The enzymes elevated at 42℃ heat exposure and displayed injuries of myocardial cells. Hsp60 expression level was fluctuated during heat stress and decreased significantly after 20 min, then elevated at 120 min and reduced at 360 min of heat stress. The damages of myocardial cells characterized by enzyme elevations and over consumption of Hsp60 concerned to the functional disorder of myocardial cells at early stage of heat stress. However the significant induction results of *hsp*60 mRNA levels ($P < 0.01$) from the beginning up to 240 min of heat stress were not consistent with the classic regulatory mechanisms. *hsf-1* mRNA level was significantly increased from 10 min of heat stress, but HSF-1 protein was not simultaneously increased, indicating that HSF-1 is not the sole regulator of Hsp60 expression.
Key words: Heat shock protein60; *heat shock protein messenger* RNA; Heat stress; Myocardial cells; Heat shock factor-1

Introduction

Stress produces deleterious effects in living organisms and suppress the growth and reduce the production in animals [1]. Studies with chronic heat stress have demonstrated altered physiological, metabolic, biochemical and cellular response in animal models and poultry[2-3]. Stressors are also responsible for eventually shock and sudden death in transported pigs and heated poultry[4].

Hsps are the most broadly distributed class of proteins and are also among the most highly conserved proteins in nature and named according to their molecular weight, for example, HSP60, HSP70 and HSP90[5]. Hsp60 is a predominantly intracellularly located protein [6].

Although stress increases the synthesis of Hsps, some Hsps such as Hsp60 are also constitutively expressed, and they play an essential role in protecting cells against stress. They are up regulated when cells are exposed to elevated temperatures or other stress[7]. This increase in Hsp expression is transcriptionally regulated. The dramatic up regulation of Hsps is a key component of the heat shock response and this up regulation is induced primarily by heat shock factor (HSF)[8].

The aims of this study were to observe the expression of Hsp60 and its corresponding mRNA levels, the expression of regulating HSF-1 and its corresponding mRNA levels in *in vitro* neonatal rat primary myocardial cells, and correlate Hsp60 expression with cellular damage resulting from heat exposure for various times and HSF-1 regulate on Hsp60 expression.

The objectives of this study were to observe the expression and corresponding mRNA levels of Hsp60. To discover expression and corresponding mRNA levels of HSF-1 in *in vitro* neonatal rat primary myocardial cells and correlate Hsp60 expression with cellular damage resulting from heat exposure for various times and HSF-1 regulate on Hsp60 expression.

Material and methods

The myocardial cells were incubated 3 days at 37℃ in DMEM medium to adopt the culture temperature. After 3 days, experimental groups containing 9-cell culture plates were placed in a humidified atmosphere with 5% CO_2 and 95% air at 42℃ and control group plates were placed in a humidified atmosphere of 5% CO_2 and 95% air at 37℃. A plate from each group was removed from incubation after 0, 10, 20, 40, 60, 120, 240, 360 and 480 min.

Tests of the activities of LDH, AST, CK, and CK-MB

The activities of LDH (A-020-1), AST (C010), CK (A032), and CK-MB (H197) were measured according to the instructions given in the commercial kits (Nanjing Jiancheng Biochemical Reagent Co., Nanjing,

China).

Western blotting

Myocardial cells treated at 42℃ were washed with PBS and lysed with sodium dodecyl sulfate (SDS)-polyacrylamide gel Laemmli sample buffer. Cell lysates were collected and boiled for 5 min. Equal amounts of protein (10 μg) were subjected to SDS-10% polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane. The membranes were blocked with 5% skim milk in TBS (20 mM Tris-HCl, pH 7.6, 137 mM NaCl) containing 0.1% Tween-20 and incubated with anti-rat Hsp60 monoclonal antibody (ab59457) for 16 h at 4℃. After washing with TTBS, the membrane was further incubated with peroxidase-conjugated goat anti-mouse IgG antibody at RT for 1 h. Then, the antibody-antigen complexes were detected using western blotting luminal reagent. The bands on the developed film were quantified using Quantity One software version 4.6.2 (Bio-Rad, USA). The density of each band was normalized to that of β-actin protein.

Detection of *hsp*60 mRNA by fluorescence quantitative real-time PCR (qPCR)

Isolation of total RNA for RT-PCR: The total RNA was isolated from heat stressed and control group myocardial cells to detect the mRNA. The Moloney murine leukemia virus (M-MLV) Reverse Transcriptase is used in the first step to extend a primer hybridized to an RNA sample. M-MLV has higher stability and lower intrinsic RNase H activity than AMV Reverse Transcriptase. Both random decamers and oligo dT are provided in the RETROscript® Kit. Catalogue number AM1710.

Primers designed for hsp60 mRNA: Primer sets were specifically designed to anneal each target mRNA, and the sequences of *hsp*60, *hsf*-1, and *β-actin* mRNA were obtained from the National Center for Biotechnology Information (NCBI) GenBank database (accession numbers NC_005108.2, NP_077369.1, and NC_005111.2, respectively). Then, primers were designed using Primer Premier 5.0 software for conventional and RT-PCR amplification.

Results and discussion

The levels of AST, LDH, CK, and CK-MB in the cellular supernatant

AST activity was obviously induced in the heat-stressed groups ($P < 0.01$) in comparison with the control levels throughout the heat stress period. LDH activity displayed the same induction tendency as AST levels, although statistically significant induction was only observed after 20 and 480 min of heat stress ($P < 0.05$) (Fig. 1). The levels of CK-MB were immediately increased in response to heat stress and high levels of CK-MB activity were observed after 60 ($P < 0.05$) and 120 min ($P < 0.05$) of heat stress (Fig. 1).

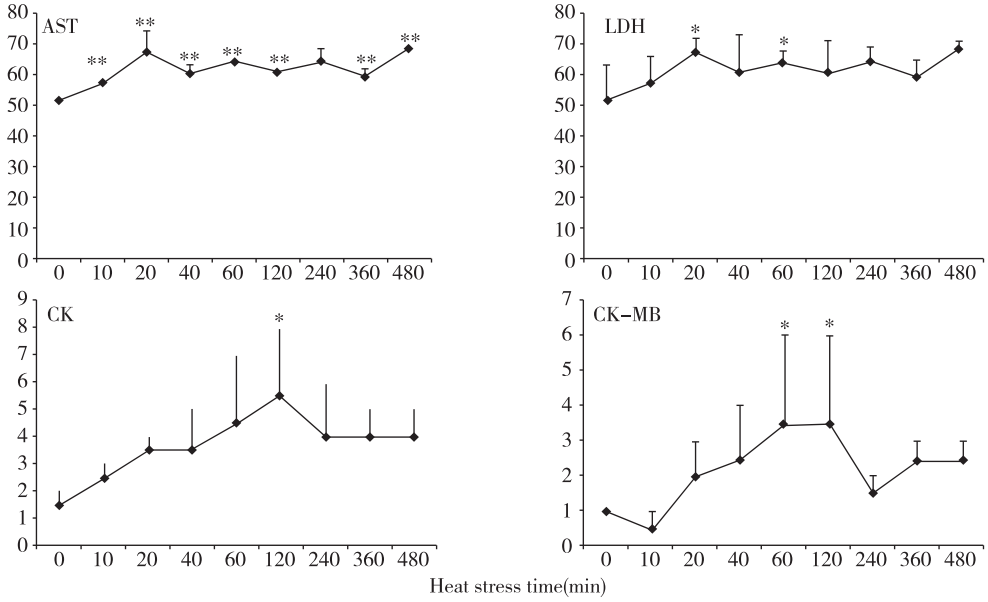

Fig. 1 Activity levels of serum enzymes in the supernatant rat neonatal cardiac myocytes (U/L)
*** $P < 0.05$; ** $P < 0.01$; Values indicated are Mean ± SD.**

Many studies reported that increased levels of AST, ALT, LDH, GGT, and CPK are consistently correlated with cardiac infarcts [9, 10]. During myocardial infarction, CK and CK-MB levels are elevated, and this

elevation plays a role in diagnosis, especially in myocarditis[11].

The transcription levels of *hsp*60 and *hsf*-1 mRNA in the heat-stressed myocardial cells

The levels of *hsp*60 mRNA in the rat myocardial cells *in vitro* were increased by heat exposure, with significant increases observed after 20 min of heat stress ($P<0.01$) and after 240 min of heat stress ($P<0.01$). Its transcription was significantly lower after 360 min of heat exposure ($P<0.01$); although its transcription remained obviously higher than that in control myocardial cells ($P<0.01$) (Fig. 2). The levels of Hsp60 remain elevated above control levels after 3, 5 and 10 h of heat exposure[12]. The transcription levels of *hsf*-1 mRNA were significantly increased after 10 min of heat stress ($P<0.01$) and sustained for 480 min of heat stress ($P<0.01$) (Fig. 3).

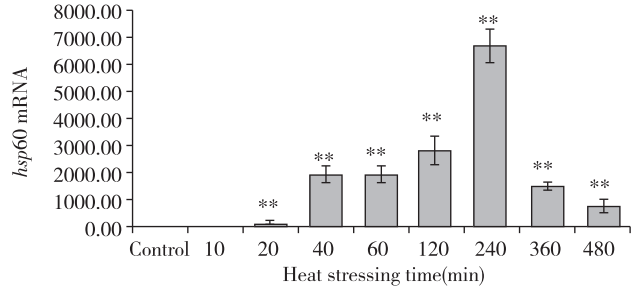

Fig. 2 The transcription levels of *hsp*60 mRNA in primary cultured cardiac myocytes of neonatal rat exposed to various heat stressing time

**** $P<0.01$; Values indicated are Mean ± SD.**

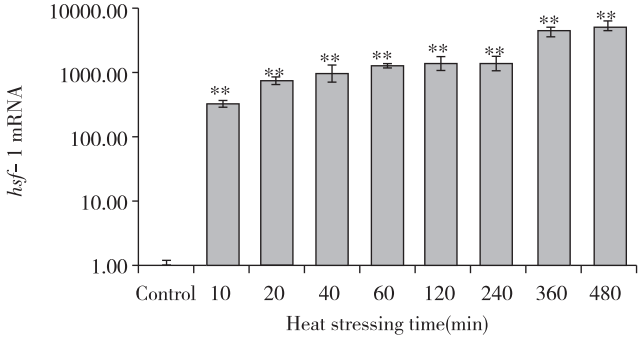

Fig. 3 The transcription levels of *hsf*-1 mRNA in the primary cultured myocardial cells of neonatal rat after heat stress

*** $P<0.05$; ** $P<0.01$; Values indicated are Mean ± SD.**

Quantitative detection of Hsp60 and HSF-1 expression in the heat-stressed myocardial cells

Hsp60 expression was decreased significantly ($P<0.01$) in response to heat stress compared with that in the control group particularly at 20 and 40 min, after which it started to increase gradually (Fig. 4). After 120 min of heat exposure, Hsp60 expression was significantly increased ($P<0.01$) and sustained after 240 min of heat exposure ($P<0.05$). After this point, Hsp60 expression started to decrease gradually and its expression was significantly lower as compare to control cells after 360 and 480 min of heat stress ($P<0.01$) (Fig. 4).

As compared to the control group levels, the levels of HSF-1 were obviously decreased ($P<0.01$) in response to heat stress for up to 120 min. Beginning at 240 min of heat exposure, the levels of HSF-1 gradually and significantly increased and remained elevated after 480 min of heat stress (Fig. 5). Our results are in line with those revealing that the levels of *hsp*60 mRNA transcription in the heart decrease sharply after 3 h of heat stress[13]. When the Hsp protein concentration increases to a high level, it can bind HSF, thereby inhibiting HSF activation and reducing the HSF- and heat shock element (HSE)-specific binding that controls heat shock gene transcription. Consequently, *hsp* mRNA transcription can be sustained at a certain level[14].

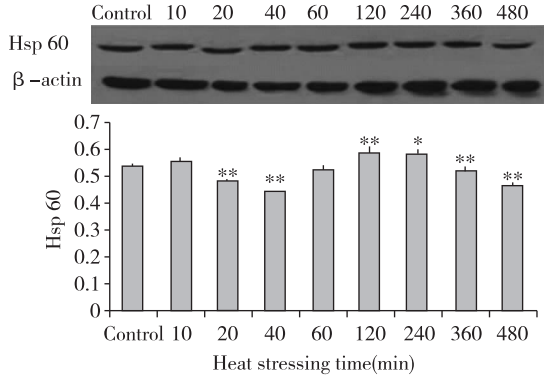

Fig. 4 Expression levels of Hsp60 in the primary cultured myocardial cells of neonatal rat after heat stress

*** $P<0.05$; ** $P<0.01$; Values indicated are Mean ± SD**

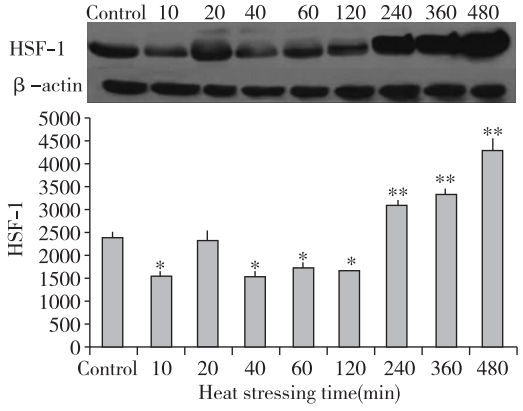

Fig. 5 Expression levels of HSF-1 in the primary cultured myocardial cells of neonatal rat after heat stress

*** $P<0.05$; ** $P<0.01$; Values indicated are Mean ± SD**

Conclusion

It can be concluded that elevated Hsp60 expression protects myocardial cells by assisting in the folding/unfolding of different proteins. The release of myocardial enzymes showed the damage of myocardial cells to certain degree. The protein expression was decreased first and then increased to protect the cells against heat stress but decreased after 240 minutes of heat stress and indicated that cells have exhausted. But the expression of HSF-1 is remained high during stress treatment and showed the production of HSF-1. However, the detailed mechanism is still unclear and requires further study.

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Localization and Semi-quantitative Analysis of Hsp110 and HSF-1 Expression in Primary Rat Myocardial Cells Exposed to Heat Stress *in vitro*

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Summary: This study aimed to understand the mechanisms of heart failure-induced sudden animal death during heat stress. We investigated the relationship between heat stress-induced pathological changes *in vitro* and expression of heat shock protein 110 and heat shock transcription factor-1 (HSF-1) in myocardial cells incubated at 42 C. Heat stress increased aspartate aminotransferase, lactate dehydrogenase and creatine kinase activity in the media and induced pathological changes, confirming that heat stress altered the integrity of myocardial cells. Hsp110 and HSF-1 were sensitive to heat stress in a time-dependent manner. Low levels of Hsp110 accumulated in the cytoplasm and nucleus after 60 min heat stress; Hsp110 expression significantly increased between 240 and 480 min. Nuclear HSF-1 expression initially decreased and then significantly increased between 240 and 480 min. Myocardial cell damage was most severe before upregulation of Hsp110; however, induction of HSF-1 and upregulation of Hsp110 attenuated heat stress-induced myocardial cell damage.

Key words: Heat shock protein 110; Heat shock transcription factor-1; heat stress; primary rat myocardial cells; *in vitro*

Introduction

HSPs play a role as protective proteins in the cell, and are expressed conversely and generously under physiological conditions or in cells exposed to stress conditions [1]. Hsp110 can precisely recognize denatured proteins and maintain them in a stable, soluble state to enable their return to the native conformation[2, 3]. Heat shock transcription factors (HSFs) are major activators which regulate and promote the transcription of *HSP* genes in response to heat stress[4]. The aim of this study was to understand the relationship between the formation of pathological lesions *in vitro*, and characterize the localization and expression of Hsp110 and HSF-1 in rat myocardial cells exposed to different durations of heat stress.

Material and methods

The experiment was done according to the following procedure: a) Primary rat myocardial cells were incubated at 37℃ and stressed at 42℃ for 10 min, 20 min, 40 min, 60 min, 120 min, 240 min, 360 min or 480 min; b) The levels of Aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and creatine kinase (CK) activities were detected; c) The cytopathological changes of the stressed cells were observed; d) The levels of Hsp110, HSF-1 and β-actin protein were detected by western blotting; e) The distribution of Hsp110 and HSF-1 were detected by immunofluorescent staining.

Results and discussion

In the present study, cytopathological analysis of heat-stressed primary myocardial cells demonstrated that acute degeneration and damage occurred with the increase of AST, LDH and CK activity over 480 min of heat exposure. In the present study, the variation in the intensity of Hsp110 positive immunofluorescent staining in heat stressed myocardial cells coincided with the Hsp110 protein expression level, as detected by Western blotting. In contrast to Hsp110, HSF-1 staining was only observed in the nuclei of primary rat myocardial cells, in agreement with a previous study [5]. The different patterns of Hsp110 and HSF-1 expression observed in heat stressed myocardial cells *in vitro* may have functional significance, and may reflect the processes which occur to stabilize intracellular protein structures[6]. It has also been reported that overexpression of HSPs can regulate the activation and expression of HSF-1 via a negative feedback mechanism[7]. However, the pattern of HSF-1 and Hsp110 induction observed in this study was similar between 240 min and 480 min of heat stress.

Conclusions

In conclusion, both Hsp110 and HSF-1 played differential role exposed to hyperthermia stress.

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Hsp70 and HSF-1 Expression Is Altered in the Tissues of Pigs Transported for Various Periods of Times

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Summary: The aim of this study was to assess changes of Hsp70 and HSF-1 protein and mRNA expression in stress-sensitive organs of pigs during transportation for various periods of time. Twenty pigs were randomly divided into four groups (0 h, 1 h, 2 h, and 4 h of transportation). A significant increased activity of AST and CK was observed after 1 h and 2 h of transportation. Histopathological changes in the heart, liver, and stomach indicated that these organs sustained different degrees of injury. Hsp70 protein expression in the heart and liver of transported pigs did not change significantly while it increased significantly ($P < 0.05$) in the stomach. *hsp70* mRNA levels decreased significantly ($P < 0.05$) in the heart after 4 h of transportation. However, mRNA expression increased significantly in the liver after 1 ($P < 0.05$) and 4 h ($P < 0.01$) of transportation, and increased significantly in the stomach of the transported pigs after 1, 4 ($P < 0.01$), and 2 h ($P < 0.05$). HSF-1 levels were reduced at 1 and 4 h ($P < 0.05$) only in the hearts of transported pigs. These results indicate that Hsp70 mediates distinct stress-related functions in different tissues during transportation.

Key words: heat shock factor-1, heat shock protein 70, pig transport, stress-sensitive organs, transport time

Introduction

Transport-induced stress can affect pigs in multiple ways, such as weight loss or impaired weight gain, decreased conversion of feed into meat, increased physical injury, and susceptibility to disease, which can lead to reduced meat quality [1]. Mammals generally respond to pathophysiological insults at the molecular level by increasing intracellular levels of heat shock proteins (Hsps) [2]. Hsp70 is one of the most highly conserved and strongly heat-inducible Hsps found in eukaryotic cells. Induction of Hsp expression in response to stress is often mediated at the transcriptional level by members of the heat shock factor (HSF) family of proteins. Mammalian genomes encode three HSF homologues: HSF-1, HSF-2, and HSF-4 [3]. The aim of this study was to assess the changes of Hsp70 protein and mRNA expression in pigs transported for different periods of time, and to define the relationship between Hsp70 and HSF-1 expression in stress-sensitive organs.

Materials and methods

A total of 20 hybrid pigs of the Erhualian and Pietrain strains were randomly divided into four groups but were kept in their individual pens until the beginning of the particular experimental transport period. The control pigs were left in their individual pens until sacrifice. The remaining three groups were transported in one commercial trailer pulled by a van for 1, 2, or 4 h at 30 – 40 km/h.

After transportation, the animals in each experimental group were euthanized either in the truck or animal shelter (control group) with 3% sodium pentobarbital (10 mg/kg body weight) delivered by jugular injection, and brought to the operation center which is near to the farms. The abdomens were immediately opened and the heart, liver, and stomach were removed for later histopathological analysis. Tissue samples to be used for subsequent evaluation of Hsp expression were placed in 1.5 mL tubes and frozen in liquid nitrogen.

Results and discussions

CK and AST activity levels in pigs transported for various time periods were higher than those of control pigs. Increased enzymatic activities of CK and AST in the blood serum are often associated with fatigue and muscular exercise during road transportation [4] as well as liver and heart disease [5,6]. Acute degeneration was also observed in the hearts, livers, and stomachs of transported pigs. These results indicated that the heart, liver, and stomach tissues sustained damage, particularly

at the beginning of transportation, and are in agreement with the findings from previous studies [7,8].

Hsp70 has previously been demonstrated to have important cytoprotective functions in the gastric mucosa both *in vitro* and *in vivo* [9,10]. High levels of Hsp70 can protect gastric cells from NH_2 Cl-induced injury [11]. Decreased Hsp70 expression causes a reduction in gastric mucosa protection and can lead to stomach tissue injuries, including ulcers [12]. Hsp70 family members can refold degenerated proteins by recognizing and binding to the cytoskeletal myosin heavy chain and actin in damaged gastric mucosa [13]. Our results showed that the levels of the Hsp70 protein significantly increased after transportation, indicating that Hsp70 also plays an important role in protecting the stomach tissues during transportation.

In the present study, variations in Hsp70 protein levels did not correspond to changes in Hsp70 mRNA expression in the hearts, livers, or stomachs of transported pigs (Table 1). These observations are not consistent with the classical transcription and translation regulatory mechanisms. However, a striking feature of heat shock genes is that expression in different organisms and types of cells within an organism can be regulated through distinct and unique mechanisms [14]. Similar to the results reported in our study, Chen et al. [15] also observed discordant Hsp70 protein and mRNA expression levels in lung adenocarcinomas. Control of Hsp70 expression appears to be regulated in a complex manner at both the transcriptional and post-transcriptional levels. Banerji et al. demonstrated that Hsp70 transcription is rapidly induced by heat shock, reaches maximal levels by 60 min, and decreases thereafter. This group also found that Hsp70 transcription increased 20-fold by 60 min and remained constant through 6 h of heat shock. These observations are similar to our findings. Cytoplasmic accumulation of *hsp70* mRNA is thought to reach a critical level that either directly or indirectly affects the rate of *hsp70* gene transcription. We hypothesize that differences in the expression of Hsp70 protein and mRNA may be due to consumption of the Hsp70 protein after playing protective functions during transportation.

Induction of Hsp expression is reportedly mediated by HSF through binding to heat shock elements (HSEs) present in the promoter regions of *hsp* genes [12]. In the present study, HSF-1 levels varied in different tissues after transportation (Fig. 1). Under normal conditions, HSF-1 localizes to the cytosol as an inactive monomer. However, HSF rapidly assembles into a trimer in response to heat shock or other physiological stresses and accumulates in the nucleus. Nuclear accumulation of HSF trimers leads to increased HSE binding, which activates the transcription of Hsps. HSF is then converted back into the monomeric form, and Hsp expression returns to basal levels. In the present study, the levels of HSF-1 did not appear to correspond to Hsp70 expression levels in different tissues. These observations suggest that Hsp70 levels in pigs during transportation may be regulated by factors other than changes in HSF-1 expression.

A previous study showed that Hsp72, which is a number Hsp70 family, is constitutively expressed in all portions of the swine heart and that expression of this protein may not be dependent on an HSF: HSE interaction. In contrast, Abravaya et al. [1] reported that transcriptional regulation of the human *hsp*70 gene in response to heat shock and other forms of physiological stress occurs through activation of HSF. Arimoclomol, an experimental drug that activates the heat shock response, can prolong activation of HSF-1, resulting in an increase of Hsp70 and Hsp90 expression in *SOD1* ^{G93A} mice. However, Hsp70 can also negatively regulate HSF-1 activation. Taken together, these results suggest that the regulatory mechanisms linking HSF-1, HSE, and Hsp70 are very complex. Whether the activation of HSF-1 was prolonged or negatively regulated by Hsp70 in the present study remains unclear and requires further examination.

Conclusions

Our experiments results indicate that the stress-sensitive organs (heart, liver and stomach) of pigs sustained different degrees of injury during transportation. Hsp70 mediates distinct stress-related functions in different tissues during transportation.

Table 1 Changes in Hsp70 protein and mRNA levels in tissues from pigs transported for different periods of time

		Control group (n = 5)	Transported groups (n = 5)		
		0	1	2	4
Heart	Protein	11.28 ± 2.98	14.36 ± 3.59	11.54 ± 1.07	15.42 ± 1.81
	mRNA	1.23 ± 0.28	1.28 ± 0.39	1.40 ± 0.25	0.59 ± 0.17 *
Liver	Protein	17.63 ± 3.88	15.07 ± 3.44	13.11 ± 1.52	14.38 ± 3.38
	mRNA	1.14 ± 0.22	1.83 ± 0.39 *	0.90 ± 0.30	3.16 ± 0.97 *
Stomach	Protein	8.62 ± 0.29	10.56 ± 0.95 *	10.19 ± 1.62 *	10.19 ± 0.7 *
	mRNA	1.38 ± 0.34	10.19 ± 2.15 * *	3.21 ± 0.75 *	6.58 ± 1.79 * *

* $P < 0.05$ and * * $P < 0.01$ compared to the 0 h. Values represent the mean ± SD.

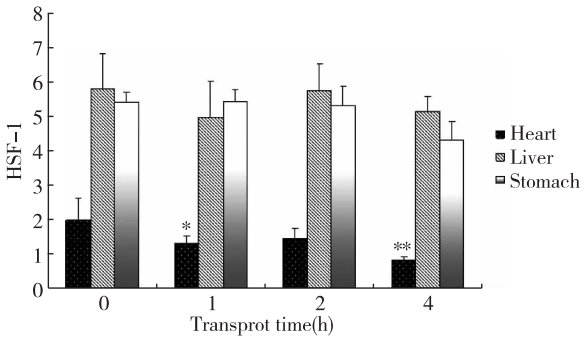

Fig. 1 HSF-1 protein levels in the heart, liver, and stomach of transported pigs
*** P < 0.05 and ** P < 0.01 compared to the Oh. Values are presented as the mean ± SD.**

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Ultrastructural Aspects of *Perkinsus olseni* under Host Encapsulation

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Summary: *Perkinsus olseni* is a unicellular protozoan parasite affecting a number of commercially important mollusks and is associated with mass mortalities. The ultrastructural features of *Perkinsus olseni* under host encapsulation were studied using transmission electron microscopy. The overall ultrastructural features observed in this study were similar to the previously described features of the cultured parasite cells and the cells from infected host tissues. However, several new features are further founded and definitely worth noting. In the nodule, the parasites were under host encapsulation, and the integrity of the plasma membrane of the parasite was maintained as normal cell, but the organelles of the cell exhibited changes, including missing membranes of the organelles, condensation of the nucleus and the cytoplasm, the increasing number of vacuoles in cytoplasm, the appearance of bilayer-organelles which similar to spliceosomes. These morphological changes were consistent with the typical characteristics of cell apoptosis which indicated that this protozoan might undergo programmed cell death as some other unicellular protozoan parasites. This is the first observation of programmed cell death in *Perkinsus* and might provide new potential targets for the design of strategies for elimination of these parasites.

Introduction

Perkinsus olseni is a unicellular protozoan parasite affecting a number of commercially important mollusks and is associated with mass mortalities. The ultrastructural features of *Perkinsus olseni* under host encapsulation were studied using transmission electron microscopy. The overall ultrastructural features observed in this study were similar to the previously described features of the cultured parasite cells and the cells from infected host tissues. However, several new features are further founded and definitely worth noting. In the nodule, the parasites were under host encapsulation, and the integrity of the plasma membrane of the parasite was maintained as normal cell, but the organelles of the cell exhibited changes, including missing membranes of the organelles, condensation of the nucleus and the cytoplasm, the increasing number of vacuoles in cytoplasm, the appearance of bilayer-organelles which similar to spliceosomes. These morphological changes were consistent with the typical characteristics of cell apoptosis which indicated that this protozoan might undergo programmed cell death as some other unicellular protozoan parasites. This is the first observation of programmed cell death in Perkinsus and might provide new potential targets for the design of strategies for elimination of these parasites.

Perkinsus species are important destructive unicellular pathogens of mollusks, causing tissue inflammation and mass mortalities of shellfish, especially bivalve mollusks, in various parts of the world [1]. Because of its important ecological and economic impacts, outbreaks of perkinsosis are notifiable to the World Organisation for Animal Health (formerly the Office International des Epizooties). To detect the pathogenesis mechanisms of the parasite, host-parasite interaction has always been focused, especially the interacting between parasite and host immunity system. For the lack of adaptive immunity in mollusks, innate immune responses play the main role on combat pathogens. The innate immune system comprises humoral and cellular immune mechanisms. Humoral mechanisms involve lysosomal enzymes, agglutinins, lectins and antimicrobial peptides. Cellular mechanisms appear to be the primarily responsible for shellfish immune processes and are mediated by two general classes of hemocytes: hyalinocytes and granulocytes. Hemocytes are observed in the hemolymph and interstitial spaces, and are involved in the processes of inflammation, phagocytosis, nodule formation, encapsulation, pearl formation, atrophy, necrosis, tissue liquefaction, wound repair, and oxidative burst activity. The nodular type of inflammatory response is usually observed when numerous small particles must be phagocytosed. Hemocytes are aggregated to small to large clusters of immunocytes in the circulation and further form nodule. The encapsulation has been proposed as an abortive attempt at phagocytosis of foreign bodies too large to be phagocytosed, including multicellular parasites. *P. olseni*, had been reported to be encapsulation by the host in ultrastructural studies of large foci. A non-glycosylated polypeptide secreted by the surrounding hemocytes was the main component of the

embedding material. Up till now, no research has been paid any attention to the reaction of *Perkinsus olseni* under host encapsulation.

Ultrasturctural study was widely used in the host-parasite relationship both in multicellular organisms and unicellular organisms. The ultrastructure of cultured and live *P. olseni* has been thoroughly studies a decade ago. However, these studies were majorly focused on ultrastructure of cultured parasite cells or the cell morphology in different life stage. The goal of this study was to find the *P. olseni* reaction to the host encapsulation response by transmission electron micrographs. The high qualified ultrastructure of the *P. olseni* from the nodules of the host was observed. Together with the genome information of *P. marinus*, a model was postulated to clarify these morphological features. The alignments of important molecular among the organisms offer further clues. This study provides important insight into the immune response of parasite to host immune response, and more importantly, could give a clue of new targets for the parasite elimination strategies.

Material and methods

P. olseni infected Manila clams were collected in Shandong Province in China and identified by our lab using the methods as previously described. The milky nodules were directly spliced form the soft body of Manila clams and observed by the light microscope to check the number of parasites before prepared for the transmission electron microscopy.

To detect the ultrastuctural changes of *P. olseni* under host imflammatory stress, transmission electron microscopy was used. Briefly, 2 – 4 mm diameter milky white nodules from the infected clams were collected and fixed for 2 h with 2.5% (w/v) glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.2 at 4℃. Then the material were washed twice in cacodylate buffer and post fixed for 2 h in 2% (w/v) OsO4 in cacodylate buffer at 4℃. Cells were enrobed in 1.5% (w/v) low melting point agarose solution. The blocks were dehydrated in an ethanol series and embedded in Epon. Ultrathin sections (60 – 90 nm thick) were obtained using an ultra-microtone with a diamond knife. Sections placed on copper grids were double stained with uranyl acetate and lead citrate. The grids were examined and images were acquired in a Hitachi H-7500 transmission electron microscope operated at 80 kV.

To find further evidence to support the presence of the programmed cell death in unicellular Perkinsus species, bioinformatic tools were used. The genome information of *P. marinus* was accessed from UniProtKB (http://www.uniprot.org/uniprot/). Then, the protein domain and function were predicted by SBASE (http://hydra.icgeb.trieste.it/sbase/).

Results and discussion

Under the light microscope, numerous clusters of Perkinsus Olseni cells confined within capsules were observed in the nodules, as previously described. Under transmission electronic microscopy, the overall morphological features of parasite cells in nodule also were consistent with previously study [2, 3]. For example, the parasite cells often formed clusters of 1, 2 or 4 cells (Fig. 1A, B and C). Some cells showed the typical eccentric vacuole or a signet ring-like appearance. Lipid droplets, endoplasmic reticulum, lomosomes (recognized as dense granules), mitochondria, vacuoles, and the nucleus with a prominent nucleolus could be observed clearly in some cells. Conspicuously, all the parasites checked in our experiment were totally or partially encapsulated by a non-cellular, non-fibrillar, and dense homogeneous substance with a variable profile and diameter (Fig. 1). This dense substance is considered to be secreted by granulocytes involved in the host encapsulation response, and mainly composed of a homolog of polypeptide p225. Seldom phagocytosis of *Perkinsus olseni* with encapsulation was also observed (data not shown). In that aspect, encapsulation plays a more important role in the defence against *P. olseni* in the nodules.

How the parasite reactions to the encapsulation had been neglected in previous study but interested in this study. Some phenomenon was worth noting. In Fig. 1A, although the parasite cells were with integrity cell morphology, they were stained with different intensity (cell c is darker and denser than cell a, b and d). Their appearance in the same field excludes processing and photographic differences and suggested that the cells were at different life status. Besides, more small vacuoles were present in the cytoplasm of cell c (Fig. 1A). Similar but severely dark in staining density of the parasite cells were observed in Fig. 2. No organelles could be detected in the cells and some indenting of the cell wall was appeared which indicated cellular shrinkage (Fig. 1B). The cells showed in Fig. 1C contain much more large numbers of vacuoles and were darker in cytoplasm. Since all samples were processed exactly the same way, the different densities of staining reflect differential condensation of the cytoplasm of the parasite. As proved by previously report, cytoplasmic condensation, shrinkage and the appearance of vacuoles were considered to be indications of dead and dying cells. Therefore, we inferred that parasite cells encapsulated by the host were dying, a process previously inferred to occur at the later trophozoite stage. These observations provide an interesting foundation for further study of the parasite's

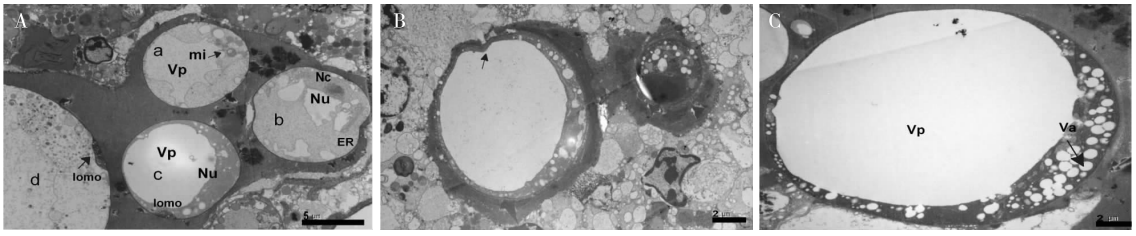

Fig. 1 Overall ultrastructural features of *P. olseni* under inflammatory stress. A, B, C represents the different *P. olseni* cells encapsulated by the host. A: Four cells, a, b, c, d were presented in one field and various organelles are present in the parasite cells: mitochondria (mi), nucleus (Nu), nucleolus (Nc), endoplasmic reticulum (ER), vacuole (Va), lomosome (lomo), vacuoplast (Vp) and cell wall (Wa). (Scale bar 5 m). B: Two *P. olseni* under the encapsulation response. Vacuoles (Va) are present in the cytoplast. C: One P. olseni cell with dark and dense in cytoplasm. The organelles of the parasite were undetectable and numerous vacuoles (Va) are apparent in the cell. (Scale bars: A:5 μm, B:2 μm, C:2 μm)

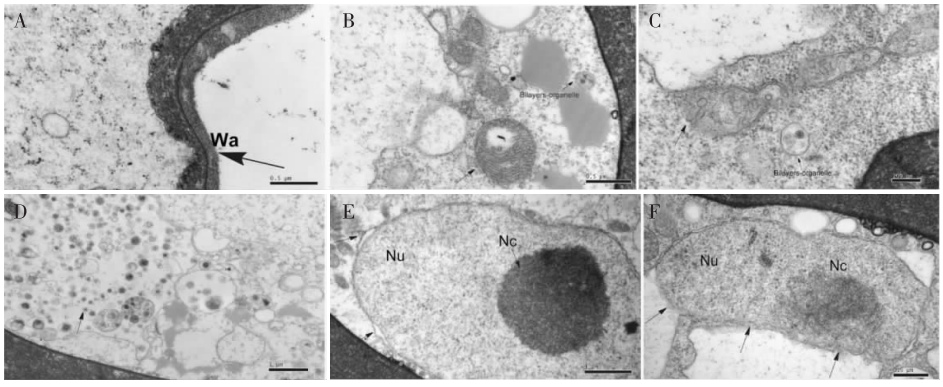

Fig. 2 Morphological features of cell membrane and the organelles in *P. olseni* under inflammatory stress. A: A representative cell membrane of *Perkinsus olseni* under encapsulation. B: Mitochondrion showing missing membrane and cristae. C: Missing membrane of the endoplasmic reticulum. A bilayer-organelle, which is perhaps an apoptotic body or autophagosome, is indicated by the arrow. D: Numerous spliceosomes and protein condensation. The arrow points to a typical bilayer-organelle. E: The nucleus. The arrows indicate nuclear membrane blebbing and rupture place. F: The nucleus. Arrows indicate the absence of the nuclear membrane. (Nc) nucleolus. (Scale bars: A:0.5 μm, B:0.5 μm, C: 200 nm, D:1 μm, E:1 μm, F:0.5 μm).

response to host inflammation stress.

The other phenomenon worth noting was that the organelles of the parasite were shown abnormal, with intact cell membrane (Fig. 2). The Fig. 2A showed a typical representation of intact of the cell membrane even at some indenting place of the cell. The distinguishable cytoplasmic organelles from parasite cells were further observed. Several mitochondria with different sizes were present in one *P. olseni* cell and parts of the mitochondrial membranes were disrupted (Fig. 2B). Additionally, some mitochondrial cristae were missing (Fig. 2B). Similarly, the membranes of the endoplasmic reticulum were disrupted too (Fig. 2C). Bilayer-organelles containing membrane-bound granules were observed and might interpret as spliceosomes, apoptotic bodies, and autophagosomes (Fig. 2C). In other cells, numerous bilayer-organelles were appeared accompanied with condensed protein in cytoplasm (Fig. 2D). Blebs and breaks in the nuclear membrane were also present (Fig. 2E). The nuclear membrane of some cell was absent as showed in Fig. 2F. These characteristics of the oragnalles were consistent with the typical characteristics of organelles when apoptosis happened. In these papers, apoptosis was mentioned as a rapid programmed cell death characterized by cell shrinkage, membrane blebbing, altered plasma membrane permeability, exposure of phosphatidylserine (PS), loss of mitochondrial integrity, chromatin condensation, DNA fragmentation, and protein cleavage. With these results, we concluded that *Perkinsus olseni* under host encapsulation preceded the cell death, using apoptosis way.

For the role of *Perkinsus* in the host-parasite interaction, the documents were limited. It is well-known that a potential outcome of parasite-host interactions following infection is the death of both parasite and host cells. However, the process whereby Perkinsus species die under host inflammatory response has been long

neglected. For multicellular organisms, three main pathways of cell death are generally recognized: apoptosis, autophagic cell death, and necrosis. In recent years, it has become clear that unicellular parasites can also undergo programmed cell death in response to various stimuli. Such suicidal responses have been described in at least 13 unicellular eukaryotic species, including parasitic organisms of the genera Trypanosoma and Leishmania [4]. For a unicellular organism, programmed cell death leads to the death of the entire organism, which does not appear to be an efficient tactic for the survival of the parasite. Possible reasons why unicellular parasites might proceed to programmed cell death might 1) could be a strategy for controlling the parasite population that avoids premature injury or death of the host) programmed cell death by apoptosis or autophagy is reversible, which provides energy and facilitate survival for up to several days. However, if conditions do not improve, self-digestion would continue and eventually would result in autophagic cell death.

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Conclusion

Ultrastructural aspects of *Perkinsus olseni* under host encapsulation revealed that the cell was progressed apoptosis, which further indicated that this protozoan might undergo programmed cell death as some other unicellular protozoan parasites.

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Peste Des Petits Ruminants Virus Exploits the Autophagy Machinery to Facilitate its Replication in Vero Cells

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Summary: Peste des petits ruminants virus (PPRV) is an important pathogen that seriously influences the productivity of small ruminants worldwide. Although PPRV is known to induce apoptosis in infected cells, the interaction between PPRV and permissive cells requires further elucidation. Here, we provide the first evidence that PPRV infection triggered autophagy in Vero cells based on the appearance of abundant double- and single-membrane vesicles, the accumulation of LC3 fluorescent puncta, the enhancement of LC3-Ⅰ/-Ⅱ conversion, and autophagic flux. We further demonstrated that induction of autophagy with rapamycin significantly increased PPRV progeny yield and nucleocapsid (N) protein expression, while inhibition of autophagy with siRNA targeting *ATG7* resulted in diametrically opposite results. Our data indicate that PPRV exploits the autophagy machinery to facilitate its own replication in host cells, thus the production efficiency of live attenuated PPRV vaccines may be improved by targeting the autophagic pathway.

Introduction

Peste des petits ruminants (PPR) is an acute, febrile, highly contagious infectious disease of domestic animals caused by peste des petits ruminants virus (PPRV), which predominantly infects small ruminants such as sheep and goats (Banyard et al., 2010). For the control of PPR, the first homologous vaccine against PPR was developed by attenuating the Nigeria 75/1 isolate through serial passage on Vero cells. However, to date, the underlying molecular mechanisms that affect the replication of PPRV in permissive cells are still poorly documented and require further exploration.

Material and methods

Cell line and virus

Vero cells were cultured in DMEM supplemented with 10% fetal bovine serum. The PPRV vaccine strain, Nigeria 75/1, was obtained from the China Institute of Veterinary Drug Control (Beijing, China).

Antibodies and reagents

The monoclonal antibody (McAb), 5B11, raised against the N protein of PPRV, was a kind gift of Dr. Jun Ai from the Yunnan Entry-Exit Inspection and Quarantine Bureau, China.

Virus infection and cell treatment

Vero cells were infected with PPRV Nigeria 75/1 at an MOI of 1 $TCID_{50}$ per cell, or mock infected with sterile phosphate-buffered saline (PBS).

Transmission electron microscopy (TEM)

Vero cells were mock infected or infected with PPRV Nigeria 75/1 at an MOI of 1 $TCID_{50}$ per cell for 48 h. Then cell samples were processed for TEM analysis as previously described (Zhang et al., 2011).

Western blot analysis

Protein sample preparation and western blot analysis were performed as previously described (Zhang et al., 2011).

Confocal immunofluorescence microscopy

Vero cells were seeded on the coverslips grown in six-well cell culture plates (Corning, NY). Cells grown to approximately 70% confluence were mock infected or infected with PPRV at an MOI of 1 $TCID_{50}$ per cell for 48 h. Then cell samples were processed for immunofluorescence microscopy analysis as previously described (Zhang et al., 2011).

RNA interference

Vero cells grown to 60% – 70% confluence in six-well cell culture plates were transiently transfected with *ATG7* siRNA using Lipofectamine 2000 (Invitrogen, Hercules, CA), as previously described (Zhang et al., 2011).

$TCID_{50}$ assay

Total virus yields were determined by the microtitration infectivity assay, calculated using the Reed-Muench method, and recorded as $TCID_{50}$/mL.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Differences were considered statistically

significant at $P < 0.05$.

Results and discussion

Autophagy is upregulated in Vero cells infected with PPRV

As shown in Fig. 1, a great number of single- and double-membrane vesicles were generated in the cytoplasm of PPRV-infected Vero cells (Fig. 1 B – E). In contrast, a significantly lower number of similar vesicles were observed in mock-infected Vero cells (Fig. 1 A). Further quantitative analyses revealed that there was a significant increase in the number of autophagosome-like vesicles in the cytoplasm of Vero cells upon PPRV infection (Fig. 1 F).

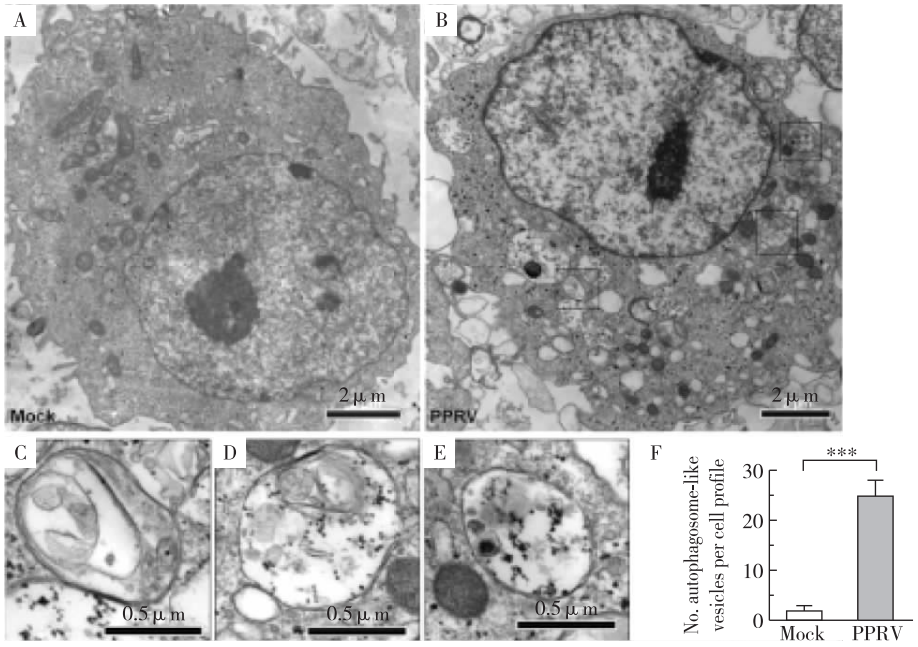

Fig. 1 PPRV infection triggers the formation of autophagosome-like vesicles in Vero cells

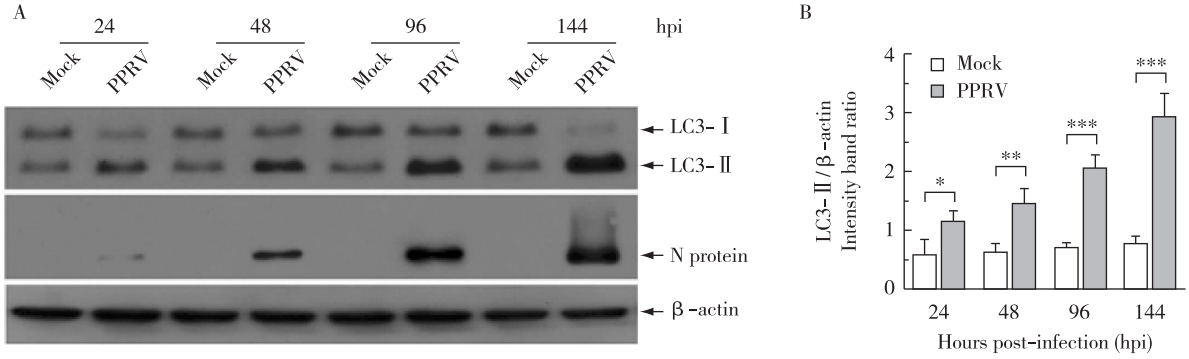

Fig. 2 PPRV infection increases the conversion of LC3- Ⅰ to LC3- Ⅱ in Vero cell

To further confirm whether the observed autophagosome-like vesicles were indeed related to autophagy, western blot analyses were performed to detect the conversion of LC3- Ⅰ to LC3- Ⅱ. As shown in Fig. 2 A, the bands of LC3 protein in PPRV-infected Vero cells displayed a characteristic conversion as infection progressed, where the band intensity of LC3- Ⅰ gradually decreased to undetectable while that of LC3- Ⅱ gradually increased. In contrast, the band intensity of both LC3- Ⅰ and LC3- Ⅱ in mock-infected Vero cells underwent no obvious changes. Further quantitative analyses showed that there was a statistically significant increase in the densitometric ratio of LC3- Ⅱ to β-actin in Vero cells upon PPRV infection from 24 h post-infection (hpi) onward (Fig. 2 B).

As an additional confirmatory test, the confocal immunofluorescence analysis showed that PPRV infection resulted in a significant enhancement of punctate staining signals of LC3 (green) distributed throughout the entire cytoplasm (Fig. 3 B, panel e), whereas the mock-

infected Vero cells exhibited a faint diffuse staining pattern and showed no LC3 punctate accumulation (Fig. 3 A, panel a). More importantly, signals of N protein (red) in PPRV-infected cells also displayed punctate accumulation (Fig. 3 B, panel f), and the red fluorescent punctate staining of N protein were highly co-localized with the green fluorescent punctate staining of LC3 (Fig. 3 B, panel h). Taken together, these results clearly demonstrate that autophagy was significantly upregulated in Vero cells upon PPRV infection.

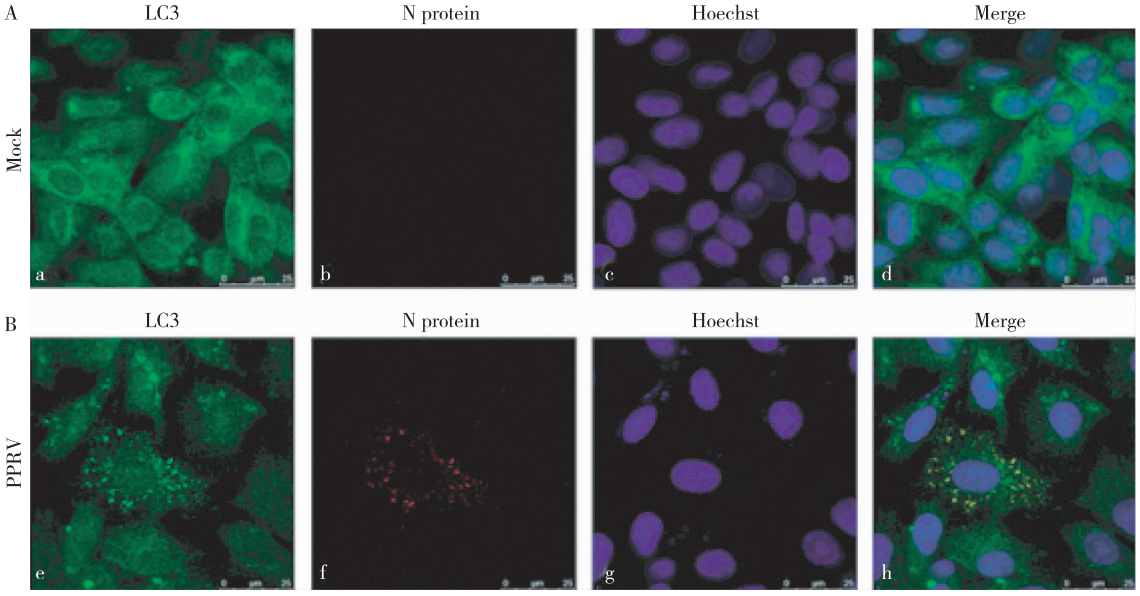

Fig. 3 PPRV infection induces the redistribution of LC3 staining signals in Vero cells

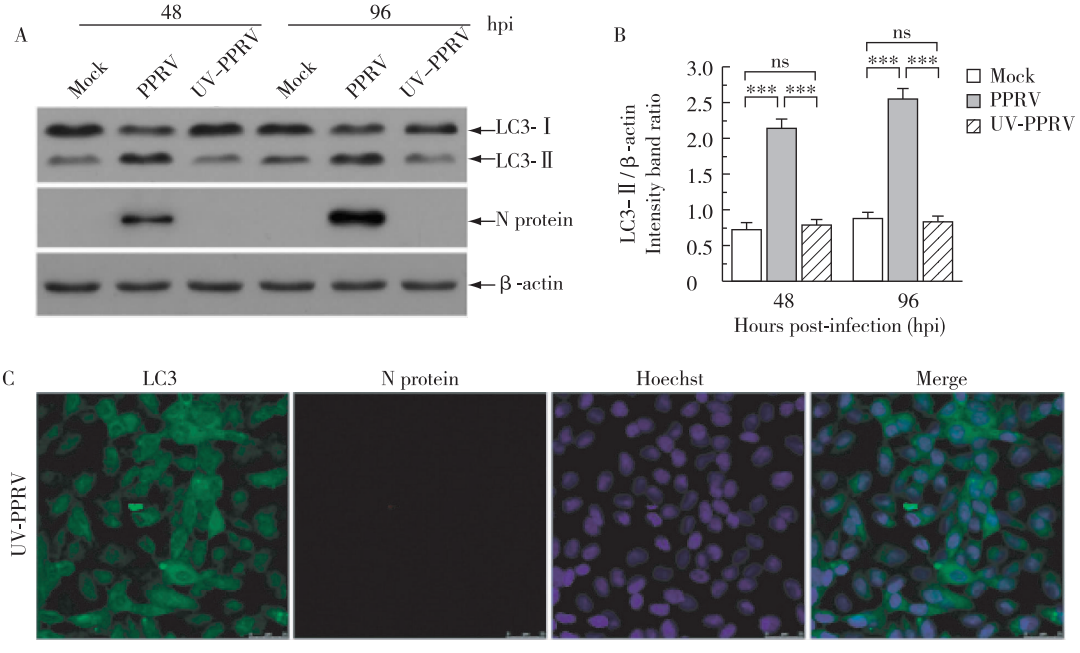

Fig. 4 The replication of PPRV is required for autophagy induction in Vero cells

Replication of PPRV is required for induction of autophagy

To explore whether viral replication is required for the induction of autophagy, live (replication-competent) PPRV was inactivated by ultraviolet (UV) irradiation and its capability for inducing autophagy was determined. As shown in Fig. 4 A, the levels of both LC3-Ⅰ and LC3-Ⅱ in Vero cells inoculated with UV-inactivated PPRV resembled those in mock-infected cells at those time points post-treatment. In contrast, LC3 protein in live

PPRV-infected Vero cells apparently underwent conversion from LC3-Ⅰ to LC3-Ⅱ, which was accompanied by a progressive increase in N protein synthesis. These data suggest that the replication of PPRV is required for the induction of autophagy.

PPRV infection enhances autophagic flux

As shown in Fig. 5, pretreatment of Vero cells with rapamycin significantly increased the amount of LC3-Ⅱ and its relative ratio to β-actin in these cells, as compared with mock-infected cells (Fig. 5 A and B), revealing that autophagosome formation was elevated upon rapamycin treatment. When rapamycin-pretreated cells were subsequently treated with E64d, the amount of LC3-Ⅱ and its relative ratio to β-actin further accumulated in these cells (Fig. 5 A and B), further verifying that rapamycin does indeed enhance the autophagic flux. Compared with mock-infected Vero cells, the amount of p62 and its relative ratio to β-actin in PPRV-infected cells significantly decreased at the indicated time points (Fig. 5 A and C), which is similar to those found in rapamycin-pretreated cells at the same time points (Fig. 5 A and C). Besides, the confocal microscopic analysis further showed that the staining pattern of LAMP1 in PPRV-infected Vero cells also presented as a discrete punctate distribution (Fig. 5 D, panel f). More importantly, a fraction of LAMP1 staining signals were highly co-localized with punctate LC3-positive fluorescent staining (Fig. 5 D, panel h). In contrast, mock-infected cells displayed weak diffuse staining for both LC3 and LAMP1 (Fig. 5 D, panels a and b). These data suggest that PPRV infection significantly enhanced the autophagic flux.

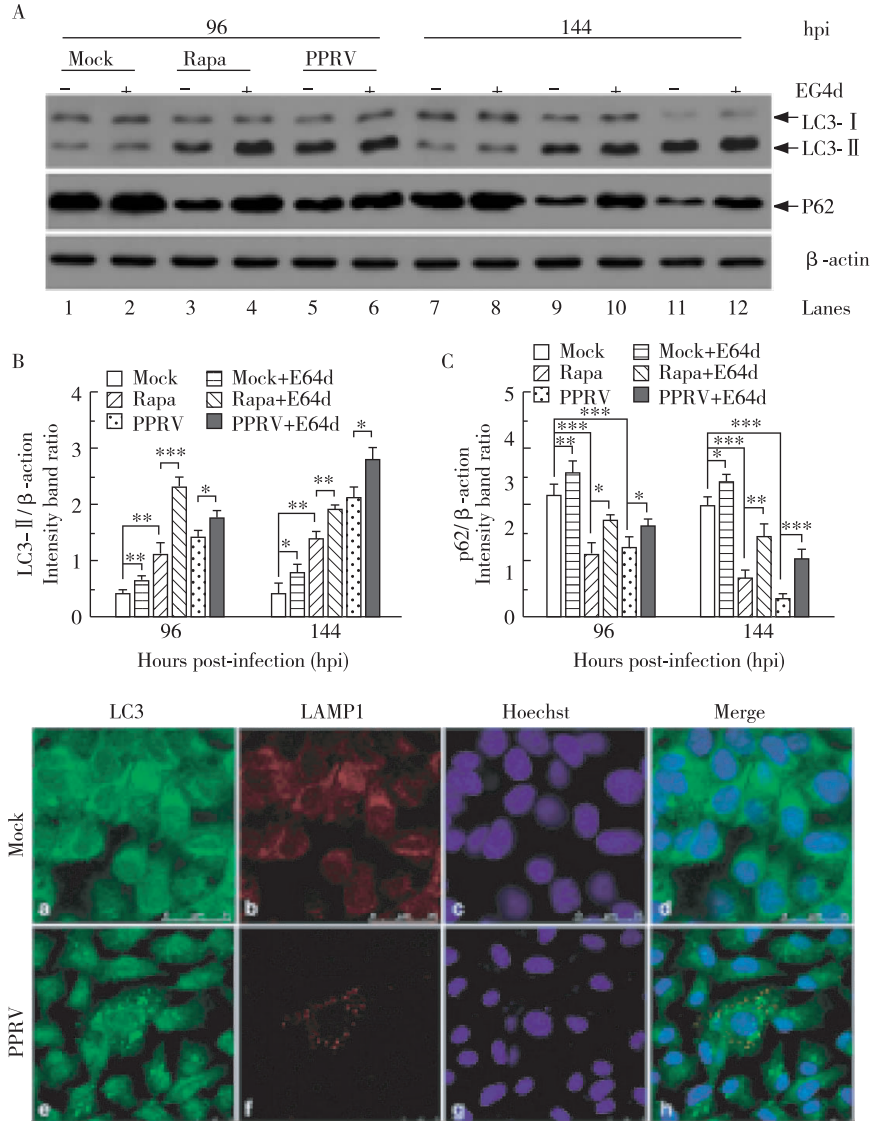

Fig. 5 PPRV infection enhances autophagic flux

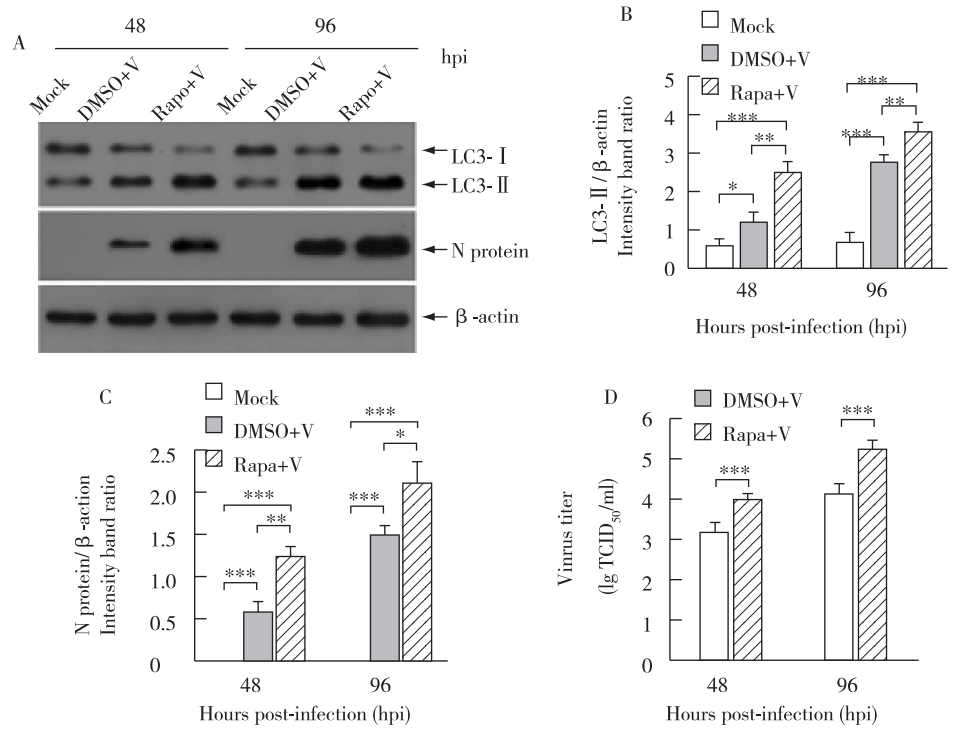

Fig. 6 Induction of autophagy with rapamycin increases PPRV replication in Vero cells

Induction of autophagy enhances PPRV replication

To explore the effect of autophagy on PPRV replication, we first investigated the effect of autophagy induction on the replication of PRRSV in Vero cells. Compared with mock-infected Vero cells, PPRV-infected cells pretreated with rapamycin or solvent control (DMSO) exhibited a higher level of autophagy, showing that the relative amount of LC3-Ⅱ in PPRV-infected cells pretreated with rapamycin or DMSO was significantly higher than that of mock-infected cells at the indicated time points (Fig. 6 A and B). Moreover, we further discovered that the relative amount of LC3-Ⅱ in PPRV-infected cells pretreated with rapamycin was significantly higher that of PPRV-infected cells pretreated with DMSO (Fig. 6 A and B), indicating that rapamycin pretreatment enhanced autophagy in Vero cells. Along with the enhancement of autophagy in PPRV-infected Vero cells, the expression of PPRV N protein was also notably increased following pretreatment with rapamycin (Fig. 6 C). Additionally, we also observed a significant increase in progeny virus yields in PPRV-infected Vero cells after rapamycin pretreatment ($P < 0.001$) (Fig. 6 D). These findings reveal that autophagy facilitates PPRV replication in Vero cells.

Inhibition of autophagy reduces PPRV replication

To confirm the findings described above, we proceeded to analyze the effect of autophagy inhibition on PPRV replication by using target-specific RNA interference. Compared with cells transfected with scrambled siRNA, Vero cells transfected with *ATG7*-targeting siRNA exhibited a significantly reduced expression level of endogenous Atg7 protein (Fig. 7 A), indicating that autophagy was successfully inhibited in Vero cells. The reduction in autophagic capacity ultimately led to a significant decrease in the expression of PPRV N protein ($P < 0.05$; Fig. 7 B) as well as the yield of PPRV progeny ($P < 0.001$; Fig. 7 C).

Conclusions

The current study provides a unique example of a live attenuated PPRV vaccine strain (Nigeria 75/1) that exploits the autophagy machinery to facilitate its own replication in the permissive Vero cells. Undoubtedly, our study pioneers a new approach to further improve the production efficiency of live attenuated PPRV vaccines that may enhance their immune effect by targeting the autophagic pathway.

Acknowledgements

This work was supported by the Basic Research Expenditure from the Chinese Academy of Inspection and Quarantine (Grant No. 2012JK011), the science and technology plan project from the General Administration of Quality Supervision, Inspection and Quarantine (Grant No. 2013IK054), and the National Key Technology R&D Program of China (Grant No. 2013BAD12B01).

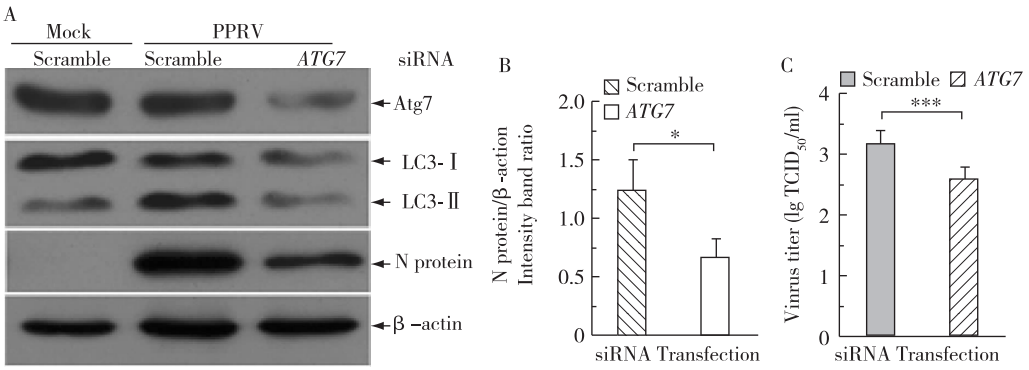

Fig. 7 Inhibition of autophagy with *ATG7* -targeting siRNA reduces PPRV replication in Vero cells

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Newcastle Disease Virus Triggers Autophagy in U251 Glioma Cells to Enhance Virus Replication

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Abstract: Newcastle disease virus (NDV) can replicate in tumor cells and induce apoptosis in late stages of infection. However, the interaction between NDV and cells in early stages of infection is not well understood. Here we report that, shortly after infection, NDV triggers the formation of autophagosomes in U251 glioma cells, as demonstrated by an increased number of double-membrane vesicles, GFP-microtubule-associated protein 1 light chain 3 (GFP-LC3) a dot formations, and elevated production of LC3 Ⅱ. Moreover, modulation of NDV-induced autophagy by rapamycin, chloroquine or small interfering RNAs targeting the genes critical for autophagosome formation (Atg5 and Beclin-1) affects virus production, indicating that autophagy may be utilized by NDV to facilitate its own production. Furthermore, the class Ⅲ phosphatidylinositol 3-kinase (PI3K)/Beclin-1 pathway plays a role in NDV-induced autophagy and virus production. Collectively, our data provide a unique example of a paramyxovirus that uses autophagy to enhance its production.

Key words: Newcastle disease virus; autophagy; microtubule-associated protein 1 light chain 3 (LC3); phosphoinositide 3-kinase (PI3K)

Rescue of Virulent Class Ⅰ Newcastle Disease Virus Variant 9a5b-D5C1

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Abstract: The virulent class Ⅰ Newcastle disease virus (NDV) variant 9a5b was generated from a nonvirulent NDV isolate Goose/Alaska/415/91 via nine consecutive passages in the chicken air sac, followed by five passages in the chick brain. The evolutionary mechanism of virulence in the class Ⅰ NDV isolate is not fully understood. To elucidate this evolutionary mechanism, a reverse genetics manipulation specific for class Ⅰ NDV is indispensable. A full-length cDNA clone of 9a5b and the helper plasmids pCI-NP, pCI-P, and pCI-L were constructed from segments of cDNA. After these plasmids were co-transfected into BSR T7/5 cells, infectious viral particles were obtained. The rescued viruses were genetically and biologically identical to the parental strain and showed similar pathogenicity in chickens. A stable recovery method for class Ⅰ NDV was established. Reverse genetics of the class Ⅰ NDV variant 9a5b allowed for the generation of genetically altered and virulent NDV, and can be used as a foundation for research on the evolution of virulence in class Ⅰ NDV isolates.

Key words: Newcastle disease virus, reverse genetics, minigenome, helper plasmids

Construction and Characterization of *cya*, *crp* Deletion Mutants from *E. coli* Causing Goose Salpingitis and Peritonitis

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Abstract: Goose salpingitis and peritonitis are fatal disease caused by Avian pathogenic *Escherichia coli* (APEC) that usually occurs in adult goose especially female goose at laying period, because of the high mortality and morbidity accompanied with low fertility and hatching rate. It has become one of the most important diseases in the goose industry in China. To date, there is still no commercial vaccine for controlling this disease. In order to improve new way for creating effective attenuated live vaccine candidate against APEC, this study constructed APEC G8107 (O2:K89) *cya* and *crp* gene mutant strains G8109 (G8107 Δ*cya*) and G8111 (G8107 Δ*cya*Δ*crp*) which has no antibiotic resistant marker by the λ Red-based recombination system, and then compared bio-characteristics and virulence among the mutants and the parent strain. Compared with the wild type G8107, the mutant strains G8109 and G8111 grew slowly during all stages of growth curve, and showed the inability on catabolism of lactose, maltose, mannitol. To exactly reflect the virulence differences, we carried out calculating mortality, clinical scoring on organ lesion to evaluate the effect of different strains to day-old-chicks. And the results of virulence assays showed death occurred in 6 of 10 who received G8107, in 1of 10 who received G8109, and no death was occurred in G8111 group; the total clinical score on organ lesion of G8107, G8109 and G8111 group were 54, 13 and 3, respectively. The results showed that the mutant strains, especially strain G8111 were less virulent compared with strain G8107. These results indicated that mutant strains G8109 and G8111 could be selected as a good candidate vaccine to APEC.

Key words: APEC, *cya* gene, *crp* gene, mutant strains, bio-characteristics, virulence assays

Nanoparticulated Heat-Stable (STa) and Heat-Labile B Subunit (LTB) Recombinant Toxin Improves Vaccine Protection Against Enterotoxigenic *Escherichia coli* Challenge in Mouse

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Abstract: Enterotoxigenic *Escherichia coli* (ETEC) remains a major cause of diarrheic disease in developing areas, for which there is no effective vaccine available. In this study, we genetically engineered a recombinant heat-stable enterotoxin (STa) coupled to the subunit B of heat-labile enterotoxin (LTB). This fusion protein, STa-LTB, possesses a single amino acid substitution at position 14 of STa. Our data demonstrates that the enterotoxicity of STa in STa-LTB was dramatically reduced. A gelatin nanovaccine candidate was prepared using the purified STa-LTB fusion protein characterized with an entrapment efficiency of 84.88% ±6.37% and smooth spheres size ranges of 80e200 nm. Antigen-specific antibody responses against STa-LTB and STa in the sera and the intestinal mucus respectively were used to test the immunogenicity of the nanovaccine. This vaccine was further screened in mice by its ability to elicit neutralizing antibodies against STa and protect animals from the challenge with ETEC in mice. The STa-LTB nanoparticles delivered demonstrated a capacity to induce significantly higher and long-lasting antibody responses and increased immune protection against ETEC challenge relative to the control STa-LTB vaccine absorbed in conventional aluminum hydrate salt ($P < 0.01$). These results warrant the further studies of the development of a novel nanoparticulate vaccine as a broad-spectrum vaccine against ETEC infection.

Reduction of Infectious Bursal Disease Virus Replication by shRNAs Targeting the VP1 and VP2 Genes Driven by Chicken U6 Promoter

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Abstract: Infectious bursal disease virus (IBDV) causes a highly contagious and immunosuppressive disease in young chickens and results in considerable economic losses for the poultry industry. To suppress the replication of IBDV, two short hairpin RNAs (shRNAs) were designed for targeting the VP1 and VP2 genes of IBDV. Recombinant plasmids carrying each shRNA or two shRNAs were constructed based on vector pSilencer2.1-U6 in which the human U6 promoter was replaced with chicken U6 promoter. In chicken embryo fibroblasts, transfection with these shRNA plasmids 24 h before infection with IBDV B87 reduced 50% tissue culture infectious doses (TCID50) from by 108.75 TCID50/0.1 mL to 103.75 – 101.0 TCID50/0.1 mL. In 10-day old specific pathogen-free (SPF) chicken embryos, incubation with a mixture of IBDV B87 and a shRNA plasmid via the allantoic cavity resulted in 100% mortality and high IBDV virus titer in the control group but 25% – 0% mortality and near normal embryo development in the specific shRNA groups; additionally, IBDV VP1 and VP2 mRNA levels were reduced by 72% – 95% in the shRNA groups as compared with the control groups. When challenged with a virulent strain IBDV GX8/99, 12 day-old chickens pre-treated with the single shRNA plasmids or the dual shRNA plasmid showed approximately 70% or 90% survival at 5 days post-challenge while those pre-treated with control plasmid or saline had less than 5% survival. The current study suggests that two IBDV shRNAs expressed by a plasmid under chicken U6 promoter could effectively and synergistically reduced IBDV replication *in vitro* and *in vivo*.

Expression in Insect Cells and Antigenic Analysis of Recombinant Nucleocapsid Protein of Canine Distemper Virus

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Abstract: The nucleocapsid (N) protein gene of an atypical Canine distemper virus (CDV) was cloned into pFastBacHTA donor plasmid. The recombinant donor plasmid pFastBac-N containing N gene was constructed and transformed into competent *E. coli* DH10Bac cells that were grown on LB plate containing 3 antibiotics. Recombinant Bacmid DNA (rBacmid-N) was obtained and used to transfect insect cells Sf9 with Lipofectamine to produce baculovirus vBacmid-N. The expressed recombinant N protein band of approximate 62 kDa was detected in Western blot. The recombinant vBacmid-N antigen was visualized in infected Sf9 cells in indirect immunofluorescence assay. Purified recombinant N protein was used to establish indirect ELISA for the detection of antibody against CDV. The absorbance values at 450 nm of all CD-positive serum samples were above 0.4 whereas CD-negative serum samples were below 0.05.

Establishment of the Hybridomas Secreting Monoclonal Antibodies Against Canine Distemper Virus

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Abstract: The splenocyte of BALB/c mice immunized with purified canine distemper virus (CDV) were fused with SP2/0 cells. Twelve hybridomas secreting monoclonal antibodies (mAbs) against CDV, designated as 3H11, 1D7, 2C9, 1F8, G12, F7, H4, A2, 2G3, 2D9, 3G12 and 3G3, were selected with inderect ELISA and cloned by the method of limiting dilution. Monoclonal antibodies secreted from hybridomas 3H11, 1D7, 2C9, 1F8, G12, F7, H4, A2, 2G3, 2D9, 3G12 and 3G3 were identified to be subclass antibodies IgG2bκ, IgG1κ, IgG2bκ, IgG1κ, IgG1κ, IgG1κ, IgG1κ, IgG1κ, IgG1κ, IgG1κ, IgG1κ and IgG1κ. ELISA titers of the acsites were 106 – 109. Indirect immunofluorescence assay (IFA) showed that the twelve mAbs could combine with the natural CDV specifically. In western blotting, mAbs 1F8 and 3G3 could react with protein N of CDV. Neutralization test indicated that mAbs 3H11, 1D7, G12, F7, H4, A2, 2G3 and 2D9 had neutralizing ability to CDV, with the neutralizing titres of 104 – 106. Addition ELISA revealed that the five mAbs could recognize the different antigen epitopes of CDV.

General Topics

New Direction for the Veterinary Medical Profession —Curriculum Proposal for Special Veterinary Medicine

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Summary: The veterinary medicine has challenges in the 21st century to pass the very wide study of all animal species in 5 or 6 years to cover more than 50 subjects in different fields of veterinary science for many species of animals, poultry, fish and wild animal medicine.

Here is proposal for development of veterinary curriculum in order to teach veterinary students a specialized Veterinary science according to the class or species of animal. Different Fields of studies have the type of specialty. Engineering studies, Art, Music, in Faculty of Science, in Human medicine, study one species' and within it there are many specialties.

Veterinary student study many subjects for all living animals (domestic and wild), birds and fishes. The veterinary profession has no alternative, it must respond to the desires of its publics and delivers veterinary services of the highest quality possible, or run the risk of losing the respect and support it currently enjoys.

The veterinary profession is expected to provide health services to the many classes of animals and animal species important to society. The public does not expect all individual veterinarians to be able to do all things. They accept the fact that veterinarians must limit their responsibilities to manageable levels.

To fulfill this expectation the veterinarian must have access to the most up-to-date information and technology. Large amount of information relevant to the husbandry, biology and diseases of any given class of animals or species. A background of training & experience. Intellectual and manual skills to identify a problem and solve it. To prepare for the challenges of the 21st Century, a veterinarian must study 2 years as general basic science, one year special academic subjects and 2 years special clinic subjects to a single class of animals or species.

Key words: veterinary sciences, veterinary curriculum, special veterinary medicine

Introduction

Most of the university studies of different fields as engineering, art, agriculture, natural sciences, handle and economic, fine arts, etc... have special curricula and the students graduate with specialized Bachelor or license.

Engineering students graduate with specialized Bachelor (Energy, civil, electric...). Agriculture students graduate with specialized Bachelor (General, crops, food industries, animal production...). Natural science students graduate with specialized Bachelor (Physic, chemistry, botany, zoology, geology, mathematics...)... etc.

The medical students graduate with Bachelor of medicine and surgery (of human, which is a one specie Homo sapiens of Class Mammalian.). We don't forget that dentists study 5 years only for the teeth of human!

The current study

The veterinarians study 4 to 5 years (Fig. 2) in order to have the Bachelor of Veterinary science or Veterinary medicine and we expect them to solve any epidemic or zoonotic problem in 5 classes of the animal kingdom (Fish, Amphibians, Reptiles, Aves, and Mammals, with their Orders, Families, Genus and species all about 56000 species, as in Fig. 1). It is impossible for a veterinarian to study even 100 species to understand its anatomy, physiology... and treat of diseases, theriogenology and surgery and other curriculum subjects. He needs the whole life to study all animal species.

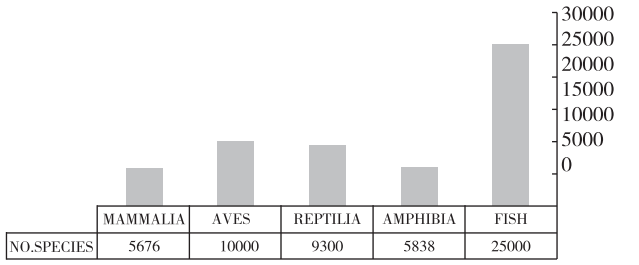

Fig. 1 No. of Chordata in which Human is one Specie out of a total of 55814 species

We have to change the focus of the profession from animal disease to animal health in all its dimensions, from the narrow viewpoint of individual animal therapeutic, to the wide point of view that emphasize on animal health and productivity as well as on disease prevention and control.

The owner is seeking advice and services on all matters relating to the animal health, The owner today wants help in choosing an appropriate pet, assistance in matters relating to pet behavior, nutrition, housing,

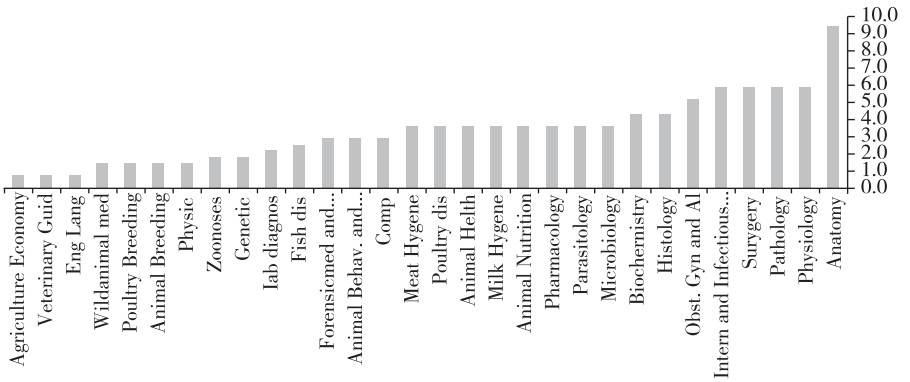

Fig. 2 Percentages of Subject total Hours in the undergraduate veterinary curriculum in Egypt 2007/2008

breeding, preventative medicine, as much or more as for the treatment of specific medical and surgical problems. Logically so, who else than a veterinarian can provide these services? Is that the self-trained and instant experts who are employed by pet stores, grooming parlors, and others?

The concept of the universal veterinarian is outmoded and should be abandoned.

The veterinary profession is expected to provide health services to the many classes of animals and animal species important to society.

The public does not expect all individual veterinarians to be able to do all things. They accept the fact that veterinarians must limit their responsibilities to manageable levels.

Time when an individual veterinarian could reasonably be expected to possess the needed skill and knowledge to acceptable to the public is long gone.

The concept of the universal veterinarian is an anachronism. It should be buried with honor. This impossible dream of the DVM who can provide for all the health needs of all creatures great and small is inhibiting the progress of the profession. It is at the very root of serious problems facing veterinary educational institutions today.

Objectives

Is to graduate veterinary students in different special Bachelor of veterinary medicine in 5 years and to prepare for the challenges of the 21st Century, a veterinarian must be: Knowledgeable about health & disease in animals in general. Have experience in depth in the application of this knowledge clinically to a single class of animals or species.

The proposed curriculum and courses

Duration of the study is 5 years. Divided into 3 stages, stage of the basic science (4 semesters), the academic stage (2 semesters), and the clinical stage (4 semesters).

All students should attend the 1st stage, the 2nd and the 3rd stages are specialized courses for each separate program.

The programs

1. Bachelor of vetmed for General program for veterinary medicine (as normal curriculum).
2. Bachelor of vetmed for Ruminant medicine.
3. Bachelor of vetmed for Equine medicine.
4. Bachelor of vetmed for Pet animal medicine.
5. Bachelor of vetmed for Wild animal medicine.
6. Bachelor of vetmed for Avian medicine.
7. Bachelor of vetmed for Aquatic medicine.
8. Bachelor of vetmed for Tropical veterinary medicine.
9. Bachelor of vetmed for Food hygiene and control.

Stage of the basic sciences (4 semesters): Obligatory and elective Courses.

1. Biotechnology and genetics.
2. Bioinformatics and statistics.
3. General anatomy and embryology.
4. General histology.
5. General biochemistry.
6. General animal behavior and management.
7. General animal nutrition.
8. General microbiology.
9. General parasitology.
10. General pathology.
11. General pharmacology and toxicology.

In addition to elective courses.

The academic stage (2 semesters): Obligatory and elective Courses according to the special program.

1. Special anatomy and embryology (e. g. ruminant or equine or pet of avian or fish or wild animal etc.)
2. Special histology.
3. Special biochemistry.
4. Special animal behavior and management.
5. Special animal nutrition.
6. Special microbiology.
7. Special parasitology.

8. Special pathology.

9. Special pharmacology and toxicology.

The clinical stage (4 semester) will contain courses according to each program.

1. Bachelor of vetmed for General program for veterinary medicine (as normal curriculum).

2. Bachelor of vetmed for Ruminant medicine.

3. Bachelor of vetmed for Equine medicine.

4. Bachelor of vetmed for Pet animal medicine.

5. Bachelor of vetmed for Wild animal medicine.

6. Bachelor of vetmed for Avian medicine.

7. Bachelor of vetmed for Aquatic medicine.

8. Bachelor of vetmed for Tropical veterinary medicine.

9. Bachelor of vetmed for Food hygiene and control.

This proposal is the extract of a study of the Veterinary Curricula in Arabic region, some Africans, Asians, European, Australian and American veterinary curricula, in the time from 2002 – 2007.

Teaching Animal Welfare with an Environmental Perspective in Mexico

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Summary: In Mexico, applied veterinary medicine encompasses the following areas: medicine and animal health, livestock production and economy, technology and food quality, public health and environmental protection. The Undergraduate Program (*Licenciatura*) in Veterinary Medicine of the Universidad Aut noma Metropolitana (UAM-Xochimilco) is organised in pedagogical modules. The module entitled "The productive animal and its environment (APA)" is laid out along the lines of Medicine and animal health, and Environmental protection. Its main objective is to propose ways to improve management and health practices and ensure the well being of animals in a specific environmental, socio-economic and cultural milieu. The three course units included in the module are oriented towards the following objectives:

1. Identify the impact of social, economic, political and cultural conditions on animal welfare, and differentiate between familiar and industrial farms.
2. Identify and evaluate cases of stress in concrete situations.
3. Propose ways to neutralise stress factors coming from management and health practices, as well as the facilities and microclimatic conditions.

In their practical work at farms and zoos, students articulate the theoretical principles so as to effect the necessary changes to the prevailing management and health practices for the improvement of animal welfare and the reduction of environmental impacts.

Introduction

In Mexico, applied veterinary medicine encompasses the following areas:

- Medicine and animal health
- Livestock production and economy
- Technology and food quality
- Public health
- Environmental protection

The Undergraduate Program (*Licenciatura*) in Veterinary Medicine of the Universidad Aut noma Metropolitana (UAM-Xochimilco) is organised in pedagogical modules. The module entitled "The productive animal and its environment" is laid out along the lines of Medicine and animal health, and Environmental protection. Its main objective is to propose ways to improve management and health practices and ensure the well being of animals in a specific environmental, socio-economic and cultural milieu. The three course units included in the module are oriented towards the following objectives:

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2. Identify and evaluate cases of stress in concrete situations.
3. Propose ways to neutralise stress factors coming from management and health practices, as well as the facilities and microclimatic conditions. This proposal seeks to cover an empty in the module APA in particular, and in the whole degree in veterinary medicine in the broad sense: the environmental dimension. This new version of the program, includes modifications, which are wide and deepens the knowledge and reflection on the environmental dimension in the course.

The objectives of the course are that graduates of the module acquire skills, attitudes and values in relation to the environment. The foregoing permits to raise the following objectives for the graduate of the module:

(a) Is capable of displaying animal production and animal welfare from an environmental perspective.

(b) Possess sufficient information on environmental issues in Mexico and various threats facing wildlife.

The course is divided into three units: the first unit, context of livestock production, gives the student a global conception of the animal relationship-environment. In the second unit, the somatic nervous system, autonomic nervous system and the neuroendocrine control are the axes of the binomial adaptation-stress (Dantzer, R. and P. Morméde. 1983). Global understanding of this system helps students infer the particular effects of the sympathetic and parasympathetic systems on specific organs. It also allows them to understand the general effects of the autonomic system.

Material and methods

The new contents of the course from the environmental perspective are:

· The ecosystem as a complex system (Clayton and Radcliffe, 1996).

· The environmental problems generated by industrial farming in the national territory.

· Models of animal production (extensive, intensive and semi-intensive) and its impact on the environment and animal welfare.

· The focus of conservation medicine.

· Animal production: capitalist (industrial) and backyard modes, its environmental impact and in animal welfare.

· Animal production with conventional and alternative species from the sustainable development (SD).

Results and discussion

The elaboration of conceptual maps of three units of the module, in which students should highlight the environmental component, facilitates them having an integrator thought.

Conclusions of the students about the environmental content of the module APA:

Diana: "the relationship between this module with the environment and sustainable development (DS), is very important. The capitalist mode of production is only concerned about economic remuneration and don't mind harming the environment."

Ariana: "the animal has to enjoy a broad, full and joyful atmosphere..."

David: "personally I changed my perspective towards the isolated concept "nature". Now I think interaction within nature, animals and humans is the important issue".

Conclusions

The experience of increasing environmental content of the module program APA can be considered positive by two factors: 80% of the students prepared the requested paper. Moreover the vast majority of students have favorable criteria to the inclusion of sustainable development (SD) issues in the program.

In their practical work at farms and zoos, students articulate the theoretical principles so as to effect the necessary changes to the prevailing management and health practices for the improvement of animal welfare and the reduction of environmental impacts.

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The Use of Bioinformatics and 3D Graphic in Teaching Veterinary Medicine

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Summary: In recent years, the rapid development of information technology and communication makes it increasingly possible and accessible to human's new ways of learning. The MILS (*Multimedia Interactive Learning System*) is the conceptual approach a teaching methodology that aims to harness the potential of the computer system. Educational technology offers us a systematic and systemic approach to analyze problems related to learning situations and to design, develop and evaluate solutions to these problems by the use and planned use of educational resources.

In developing countries the practice of education in veterinary medicine is difficult; there are often problems that limit learning as the availability of the animal and the conflict between training time and number of student.

In order to solve the problem related to learning in our institute, we have contributed to the creation of bioinformatics lab, which looks at the implementation of different projects of interactive learning and their validation.

In first time we make an interactive on avian autopsy. It is a teaching tool, that presents in a 3D application which ensures interactivity between the computer systems and learn, that was produced under the open source software Blender 3D, which has its own built in game engine that allows you to create interactive 3D applications or simulations. And the interactivity is provided by the joystick. And we have found that the application is very useful for the realization of practical avian autopsy

Introduction

Educational technology is the study and ethical practice of facilitating learning and improving performance by creating, using and managing appropriate technological processes and resources. The term educational technology is often associated with, and encompasses, instructional theory and learning theory. While instructional technology is "the theory and practice of design, development, utilization, management, and evaluation of processes and resources for learning," according to the Association for Educational Communications and Technology (AECT, 2004). Definitions and Terminology Committee, educational technology includes other systems used in the process of developing human capability. Educational technology includes, but is not limited to, software, hardware, as well as Internet applications, such as wikis and blogs, and activities. But there is still debate on what these terms mean.

Educational Technology relies on a broad definition of the word "technology." Technology can refer to material objects of use to humanity, such as machines or hardware, but it can also encompass broader themes, including systems, methods of organization, and techniques. Some modern tools include but are not limited to overhead projectors, laptop computers, and calculators. Newer tools such as "smart-phones" and games (both online and offline) are beginning to draw serious attention for their learning potential. Media psychology is the field of study that applies theories in human behavior to educational technology.

Evaluation of 3D software and game engine in veterinary teaching

1. Educational game

An educational game is a game designed to teach humans about a specific subject and to teach them a skill. As educators, governments, and parents realize the psychological need and benefits of gaming have on learning, this educational tool has become mainstream. Games are interactive play that teaches us goals, rules, adaptation, problem solving, interaction, all represented as a story. They give us the fundamental needs of learning by providing-enjoyment, passionate involvement, structure, motivation, ego gratification, adrenaline, creativity, social interaction and emotion. "Play has a deep biological, evolutionarily important, function, which has to do specifically with learning. (PRENSKY, 2001)

2. The evaluation of the use of software Blender 3D in veterinary training:

Blender is the open-source software for 3D modeling, animation, rendering, post-production, interactive creation and playback. It is available for all major operating systems under the GNU General Public License. Its animations systems support a variety of techniques and tasks, allowing the creation of complex animations. (MARKS et al, 2008)

A. Modeling and conception:

Blender has many different tools available to help for create 3D model of anatomical parts quickly and efficiently: (Fig. 1).

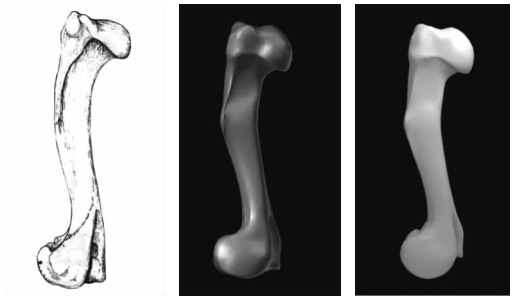

Fig. 1 Modeling and rendering the humerus bone of the dog

B. Game engine and interactive application

The Blender Game Engine oversees a game loop, which processes logic, sound, physics and rendering simulations in sequential order. The engine is written in C + +.

When creating a game or simulation in the BGE, there are four essential steps:

· Create visual elements that can be rendered. This could be 3D models or images.

· Enable interaction within the scene using logic bricks to script custom behavior and determine how it is invoked (using the appropriate "sensors" such as keyboards or joysticks).

· Create one (or more) camera to give a frustum from which to render the scene, and modify the parameters to support the environment in which the game will be displayed, such as Stereo rendering.

· Launch the game, using the internal player or exporting a runtime to the appropriate platform

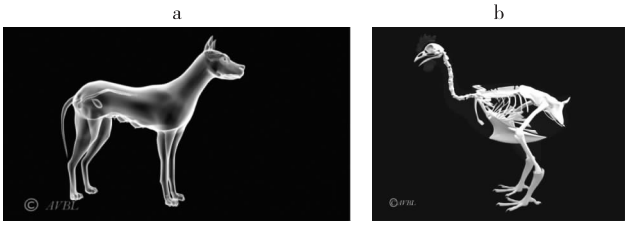

Fig. 2 a. Reproductive system of the bitch; b. Skeletal system of the chicken

3. Veterinary Bio-infographic laboratory

The creation of a laboratory for bio-infographic veterinary medicine aimed at the production of different simulator model by using 3D imaging and animation in real-time, products as images, video and applications interactive "game mode". (VICTOR, 2012) Avian interactive autopsy is an interactive education, which aims in this first version to give students a chance to perform an autopsy in real time, it mainly based on Blender 3D for the modeling of shapes and objects (Fig. 2), and even the production of the executable application (Fig. 3), in its own game engine "Blender game engine" and other open source software such as: Gimp for textures and python (API) for scripting control games with the joystick.

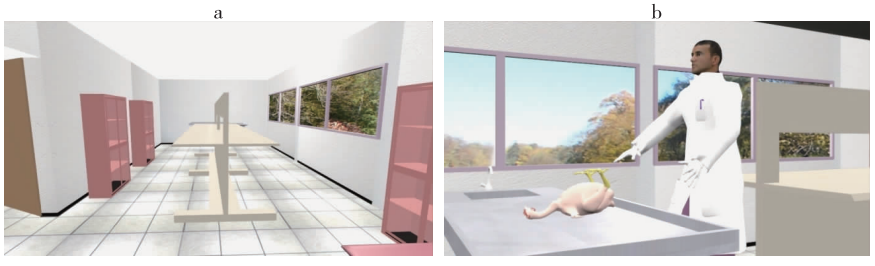

Fig. 3 a. The autopsy lab conception; b. 3D game characters

Conclusions

Interactive multimedia systems, they are pedagogical tools that use the augmented reality used in the preparation of the study in a robotic way, very close to actual medical procedures in its clinical context.

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Poster

A Typical Rabbit Production in Gwagalada Area Council of the Federal Capital Territory, Abuja Nigeria

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Summary: This paper look at the different Typical Rabbit farms in the communities of Gwagalada, in the Federal Capital Territory Abuja Nigeria. Consideration were based on the different and best practices of the cleaning, disinfection, mating, reproduction, feeding, through the different distribution, practices and analysis of the result from the field and conclusion were set in the process, with the different recommendation that was made in the process.

There are also urgent needs of information sharing between the different rabbit farmers and among, individual to foster the rabbit development in Gwagalada communities, which will also promote technology, development and marketing enhancement at all level. Combination of rabbit and other livestock were also discussed to ensure the rabbit development and technology transfer, should also need to been developed to replicate the different processes that are involve.

Conclusion were also draw through the farm visits, questions being ask and pictures that was taken to the effect.

Introduction

Rabbit generally refers to small monogastric, running animals. They are medium-sized hopping mammals, with relatively long ears and legs. They are chiefly nocturnal, have acute senses of smell and hearing. Most Rabbit live in holes; they dig under the logs or rocks. Rabbits can tolerate wide range climates from cold and wet to hot and Dry. They can withstand temperatures as low 10 F – 12℃ (Banerjee).

Gwagalada which is one of the six area council of the Federal Capital Territory, were targeted to ascertain few of the individual, rabbit farmers that are into rabbit production, in respect to cleaning disinfectant, mating, reproduction and feeding practices. More key concerns for rabbit production includes, sanitation and health, nutrition, reproduction and breeding also depend (RayMobley).

Mating and Reproduction aspect: The buck and the doe are usually kept distance apart to avoid familiarity. Most rabbit farmers allow doe to be introduced to the buck, and organized breeding programs are not common, farmers prevent indiscriminate mating. Provisions of a kindling box are not common in the rabbit farmers and individuals in Gwagalada. Separate accommodation is usually reserved for the doe (and the kits) immediately after pregnancy confirmation. Does kindle 5 – 8 kits per litter and 4 – 6 kits reach weaning age and about twenty weaned rabbits per year.

Feeds and feeding: Rabbit farmers and individual people feed their rabbits in feeding troughs, made of wooden or clay or cement, iron, metal, half-split bamboo, and empty cans. Feeds are Frequently and carefully sprinkled with little amount of water, to reduce feed dustiness and wastage. Feeds are usually not pelleted except commercially prepared diets. Diets of rabbits in Gwagalada are Primarily on forages, grasses and legumes supplemented with kitchen wastes and agricultural by-products. Such as dried cassava peels, wet milled cereal by-products. Indeed, in most rural, peri-urban and suburban of Gwagalada.

Pasture cultivation for rabbit feeding also existent in the process and green plants are also provided, despite the sole dependence on green plants. Thus, feed security becomes critical during the Dry season particularly and Rabbits lose weight during this period, and breeding is negatively affected. Forages such as Panicum maximum (guinea grass), Pennisetum purpureum (elephant grass) Tridax procumbens, sweet potato leaves, cassava leaves and groundnut haulms, and Talinium triangulare and rabbit pellets are some times given to the rabbit.

Material and methods

Different farms visits of Rabbit farmers and individual were made. The best rabbit farmers were used, to judge the best practices within the Gwagalada. Pictures were taken to support the various discussions that were made. Few questions were also asked within the rabbit's practices farmers in the different communities in Gwagalada zone of the Federal Capital Territory, Abuja Nigeria.

Results and discussion

The free range system of rabbitry

Sample is being seen in the areas of allowing the rabbits to roam about to look for grasses on the ground and also additional supplement of foods were also provided in the process. Also there are various different of males and females rabbit. Though the Doe and Buck are also being cage in the different hutches to provide different ventilations, protection, control of mating in the process.

The different hutches system

The Cleaning of the rabbit hutches is done through the process of using typical broom to remove some of the left over droppings from the rabbit and the collection of the feaces were also employed in the process.

Integrated rabbitary production

This also shows the integrated rabbit production in one of the rabbit farms respectively, observation were made in respect to the animals that are being produce. The first layers are rabbits and the second layers are pigeon production. The combination is necessary, but more reasearch needs to be conducted to the findings in respect to Feeding, housing system, health management, mangerial skills being employed and the productively level

Conclusions

Few Farmers and individuals in Gwagalada areas council of Abuja in Nigeria are into rabbit production, its has provide sources of income, but there is a particular family man, that are into the combination of rabbit production with other livestock management. As seen in the pictures of a typical rabbits farms in respect to cleaning, disinfection, mating, reproduction and feeding process. It is quite encouraging and there is a need for information sharing among the rabbit farmers and Best documentation practices and methodology development, with capacity building of the interested farmers and individual which will boost the multiplication of rabbit development process and marketing developmental strategies are therefore necessary.

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Local Immune Response of Ascarid Invasion Affected Intestine on Different Food Supplement in Chicken

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Summary: In agriculture nourishment of breeding birds plays a key role in birds' health and development of their immune system, therefore affecting quality and taste of products for consumers. The aim of this study was to evaluate the local tissue immune responses in broiler's small intestine after different food additives, focusing on the immunohistochemical detection of the cytokines and antimicrobial proteins.

Materials and methods. We analysed the small intestine of 7, 28 and 42 days old 40 broilers receiving Jerusalem artichoke root powder in different combinations with *Lactobacillus reuteri* and *Pediococcus*; 14 chicken were used for controls. The small intestine of the birds were routinely stained and analyzed also for IL-10 and antimicrobial protein beta-defensin 2.

Results revealed dramatically destructed villi as well as local prominent inflammation in intestine of all groups including the control. Moreover, massive ascarides' eggs invasion was discovered in the lymphatic and blood vessels of the intestinal wall. The Jerusalem artichoke root powder in the 0. 5% concentration decreased IL-10 producing cells on the 28th day and increased them on the 42nd day. In the *L. reuteri* group number of IL-10 producing cells decreased after 0. 5% and increased after 3% Jerusalem artichoke root powder addition in food. Food and *Pediococcus* with Jerusalem artichoke root powder on the 28th day induced decrease of IL-10 producing cells, but on the 42nd day increase of them.

Conclusions. Changes in broilers' small intestine local immune defence seems strongly depend on the invasion of the ascarides. On the 28th day 0. 5% Jerusalem artichoke root powder decreases IL-10 expression indicating the immunity inhibitory nature of this concentration powder. The local immunity response increases again at the 42 day possibly indicating both combined Jerusalem artichoke powder and bacteria positive influence and also ascaridosis stimulation of the local immunity in intestine of chicken.

Introduction

In agriculture nourishment of breeding birds plays a key role in birds' health and development of their immune system, therefore affecting quality and taste of products for consumers. One of the methods how to increase the quality of food is to enrich the nutriment with different natural additives. Feed of birds has a major role in exposing them to a variety of factors through gastrointestinal tract, therefore providing the level of effectiveness of chicken growth, egg production, and avian reproductive function [1]. During the period of incubation and first 14 days after it (newly hatched broilers' gastrointestinal tract has not totally matured) the development and increase in weight of birds' small intestine is of a greater rate than other organ systems. Early intake of exogenous nutriments and their quality has been proved to have a crucial impact on the development of small intestines [1].

From factors affecting the growth of bird immunomodulators, antibiotics and different food supplements are mentioned. To immunomodulators belong interleukins, a group of cytokines-which participate in regulation of immune processes, signal transduction between immune cells [2]. Also defensins, antimicrobial peptides that are important in innate immunity [3], produced mostly by leukocytes and epitheliocytes, influence the avian gastrointestinal tract [4].

Antibiotics are widely used in the poultry breeding to improve production and prevent birds from different diseases. However, with the increasing pathogenic, as much as commensal bacteria resistance, alternative methods are researched. A variety of probiotical bacteria, including *Bifidobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus* and different yeast species, is used [5]. Probiotics, especially the lactic acid bacteria produce antimicrobial compounds into the intestine, regulating the composition of the intestinal microflora. Probiotic bacteria are also reported to participating into developing immunity of the intestinal tract. Several authors showed that the administration of highly concentrated bacterial cultures was an effective way to promote body weight gain in chickens [6]. Oral treatment with lactobacilli is reported to modulate systemic antibody- and cell-mediates immune response, increasing the antibody production reacting on the vaccines [7]. Oral supplementation in poultry with *Pediococcus* significantly increased the relative weight of thymus, bursa and spleen, comparing to the control

group, which indicates the enhancement effect of probiotic on the systemic immunity [8].

Inulin-type fructants in poultry feeding are found to stimulate the growth or activity, or both of beneficial intestinal bacteria (like lactobacilli and bifidobacteria) and prevent colonization by pathogenic bacteria [9]. Moreover, their adding to the main food may increase the absorption of the nutrients by improving the intestinal mucosal structure, for example, by increasing villus height [9]. It can be found in many plants, especially in the root part (chicory, Jerusalem artichoke, dandelion, coneflower etc.). However, the impact on the structure from combination of inulin-type fructants and different bacteria are not researched yet. Thus, aim of this study was to evaluate the local tissue immune responses in broiler's small intestine after different food additives, focusing on the immunohistochemical (IMH) detection of the cytokines and antimicrobial proteins.

Material and methods

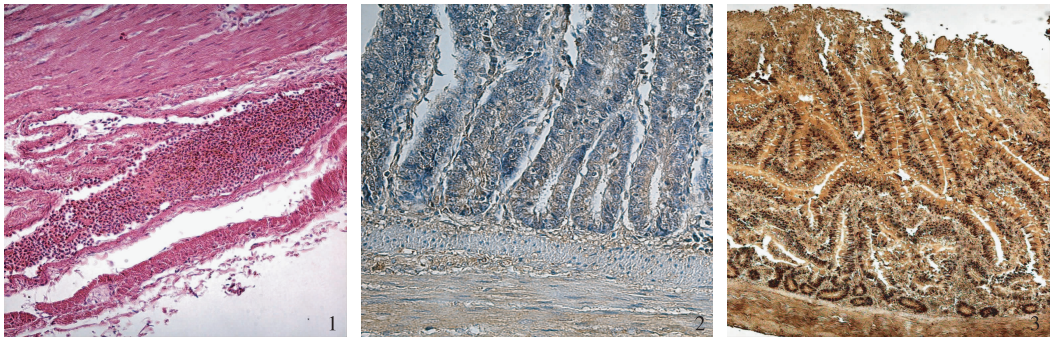
All together, we analysed the small intestine of 7, 28 and 42 days old 40 broilers receiving Jerusalem artichoke root powder in different combinations with *Lactobacillus reuteri* and *Pediococcus*; 14 chicken were used for controls.

Birds were handled according to the principles for the care of animals in poultry farm. The animal use protocol was reviewed and approved by the Animal Ethical Committee, 2010. The additives used in this experiment were Jerusalem artichoke root powder, which on the half consists of the inulin; *Lactobacillus reuteri* 1×10^8 bacteria; *Pediococcus* 1×10^8 bacteria. One-day-old broilers were obtained from a hatchery and fed a commercial starter diet for a 6-day pre-experimental period (200 g per day). On the 7th day different food additives were added to the main diet. Broilers were divided into four groups depending on the additives. In the I group were included 12 chickens, divided into two subgroups depending on the added Jerusalem artichoke root powder concentration: 0.5% (6 birds) and 1% (6 birds) Jerusalem artichoke root powder was used. In the II group to the main food was added Jerusalem artichoke root powder and *Lactobacillus reuteri*; there were included 28 birds, divides into 4 subgroups depending on the Jerusalem artichoke root powder concentration: 0.5% (7 birds), 1% (7 birds), 2% (7 birds) and 3% (7 birds). In the III group was added Jerusalem artichoke root powder and *Pediococcus*; this was divided into 2 subgroups depending on the Jerusalem artichoke root powder concentration: 0.5% (4 birds) and 1% (4 birds). The IV group was control and consisted of 14 chickens.

After the slaughtering the *ileum* of the birds were analyzed on the 7th day, when the digestive tract is fully developed and the experiment begins; on the 28th day, which is between the other two dates; on the 42th day, when all the broilers are slaughtered for the off-take. Tissues were fixed in the 10% formalin, embedded into the paraffin, sectioned at a thickness of 3 μm, and proceeded for: IL-10 (ab34843, 1∶400, Abcam, UK) and beta-defensin 2 (code 294028, 1∶500, Quartett Germany). Also haematoxylin and eosin staining was performed for each case. The semi-quantitative counting method was used for data evaluation [10].

Results and discussion

Between animals at the age of 7, 28 and 42 days the majority demonstrated a high activity of inflammation in the mucous layer of the small intestine. As our findings revealed a significant invasion with the ascarides (Fig. 1), it must be considered a factor affecting both the immune response and the inflammative changes in the intestinal wall.



Figs. 1 – 3 Micrographs of broiler chicken small intestine. 1. Intestine wall of 28 days old chicken with prominent ascarid invasion into the large blood vessel, Haematoxylin and eosin, × 250; 2. Ileum of 28 days old chicken without IL 10 cells, IL 10 IMH, × 250; 3. Intestine wall of 42 days old chicken with numerous IL 10-containing cells, IL 10 IMH, ×250.

The study of IL10 expression in small intestine in 28 days posthatch chickens demonstrated a significant decrease of IL10 producing epithelial cells(Fig. 2). Most considerable changes were observed in the group where as a nutrition additive 0.5% Jerusalem artichoke powder was used. There were no other studies found that had researched the expression of IL10 in avian small intestine epithelial cells after this specific nutrition additive, but research shows the anti inflammatory nature of this cytokine [11], therefore applied Jerusalem artichoke powders' immunity decreasing nature could be taken into consideration. In 42-day old broiler small intestine an increased production of IL10 was observed(Fig. 3) within the groups of various concentrations' artichoke powders addition to avian nutrition, which may be as a result of a correlation between Jerusalem artichoke powder and lactic acid bacteria. The recovery of local immune response indicated by the increase of anti inflammatory cytokine IL10 that was found in our study was not found to be described previously in literature.

The semi-quantitative assessment of expression of cytoplasmic beta-defensin 2 in avian small intestine showed no evident difference between birds observed at different days posthatch and different breeds. Beta-defensin 2, a small protein molecule with bactericidal and hemotactic functions, has been studied to be one of the indicators of innate immunity [12, 13] and to have a strong microbicidal activity against food-borne pathogens [13]. The expression of beta-defensin-2 in chicken digestive tract has been reported in previous studies, but in less extent than in other tissues, however its elevated expression in the proximal part of the digestive tract with lower expression levels throughout the digestive tract suggested in these reports [13] was not acknowledged in our observations that revealed a slightly more significant expression of beta-defensin 2 in the distal part of avian small intestine. Although no studies were found to have described the effects on beta-defensin-2 production after different food additives, its innate nature explains the absence of significant changes in its expression between birds of different age. In our study, an evident difference of the level of beta-defensin 2 expressions in different layers of the wall of small intestine was observed. In particular broilers' the expression was increased significantly in the muscular layer, whereas less remarkable in mucosa, which may be related with the invasion of ascarides' within blood and lymphatic vessels.

Conclusions

Changes in broilers' small intestine local immune defence seems strongly depend on the invasion of the ascarides. On the 28th day 0.5% Jerusalem artichoke root powder decreases IL-10 expression indicating the immunity inhibitory nature of this concentration powder. The local immunity response increases again at the 42 day possibly indicating both combined Jerusalem artichoke powder and bacteria positive influence and also ascaridosis stimulation of the local immunity in intestine of chicken.

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Performance of Grower Pigs Fed Green Berseem with Different Level of Garbage

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Summary: A study was conducted to evaluate the effect of different levels of green berseem, kitchen waste and concentrate feeding on growth and carcass traits in growing and finishing pigs. The experiment was conducted in two phases using 24 LWY piglets of about 3 months age. The performance of growing piglets was studied for 2 months. The details of feeding regimens in different groups were as follow, group I: 10% green berseem + 90% concentrate only, group II: 10% green berseem + 25% kitchen waste + 65% concentrate, group III: 10% green berseem + 50% kitchen waste + 40% concentrate and group IV: 10% green berseem + 75% kitchen waste + 15% concentrate. There was no significant difference between different treatment groups with respect to overall DM intake, body weight gain and FCR. However, better performance was observed for group IV. In conclusion, substitution of concentrate for different levels of kitchen waste along with 10% green berseem improves the DM intake, growth and FCR than that of the control diet. Significantly ($P < 0.01$) lowest cost of total feed intake and cost per kg live weight gain was observed for group IV followed by group III, II and I. Thus, green berseem could be incorporated up to 10% and kitchen waste up to 75% of total dry matter intake to make the feed economical and without affecting the performance of the grower pigs adversely.

Introduction

In India, livestock production systems are mainly based on low cost agro by products as nutritional inputs, using traditional technologies. In this system pig rearing fits very well which can use agro-industrial by-products such as cereal brans unfit for human consumption or high-quality forages thus may avoid competition for human foods. Under Indian condition, to optimize the growth of animals by providing them with diets of optimum quality is not the primary aim of smallholder livestock producers but the maximum economic return is normally the principal goal. Accordingly, present research has been planned to observe the effect of kitchen wastes and green pasture, in place of concentrate to the extent feasible in pig ration without affecting the performance adversely.

Materials and methods

The present experiment was designed using 24 piglets of about 3 months age. The animals were randomly divided in to 4 groups (T_1, T_2, T_3 and T_4) of 6 each. Each group had 3 replicates with two piglets in each. The performance and economics of growing piglets was studied. The feeding trial lasts for 2 months. The details of feeding regimens in different groups were as follow, group I: 10% green berseem + 90% concentrate only, group II: 10% green berseem + 25% kitchen waste + 65% concentrate, group III: 10% green berseem + 50% kitchen waste + 40% concentrate and group IV: 10% green berseem + 75% kitchen waste + 15% concentrate. The *ad libitum* feeding was done.

Results and discussion

The daily DM intake in all the groups irrespective of treatments was almost similar and there was no significant difference except at 5th week. During 5th week of the growing stage the daily DM intake was found to be significantly ($P < 0.01$) highest in group II and III followed by group IV and I. However, no significant differences were observed between group II and III. Similar reports on DMI in control and kitchen waste incorporated diet were also reported by other workers, who reported no significant difference in DMI among control and kitchen waste incorporated diets and range of DMI for grower pigs was 1.25 to 1.90 [11] and for finisher pigs was 1.97 to 2.06 [1] and 2 to 2.9 [11]. DMI for control and kitchen waste alone for grower pigs was 0.97 and 0.99, respectively. Further, results indicated that decreasing but non-significant trends of DMI were observed as the percentage of kitchen waste increased in diet of different groups. During entire growing phase (1st week to 8th week) the daily DM intake was maximum in group II in which 25% kitchen waste was incorporated followed by group III (50% kitchen waste), IV (75% kitchen waste) and lowest in group I (Control, no kitchen waste). Similar trends were also observed by [1,11]

At the end of growing stage (4th fortnight) group IV (30.32 ± 5.81) showed highest body weight gain

followed by groups III (29.27 ± 5.48), II (28.75 ± 5.56) and I (28.14 ± 5.09). However, there was no significant difference in body weight gain among different treatment groups. These findings are in conformity with the findings[5,2]. Better growth rate with kitchen waste incorporated diet might be due to high nutritious value diet which contains meat, bread, paneer, vegetable, pulses, rice etc and better digestibility of nutrients[11]. This was also supported by[6,8] who estimated crude protein value of kitchen waste to be 29.17 and 26.23%, respectively which is even more than those of NRC recommendation.

The overall average daily weight gain in groups I, II, III and IV was 505.21 ± 69.44, 552.09 ± 73.32, 538.69 ± 56.91 and 571.39 ± 66.86 g, respectively. Highest body weight gain was found in group IV followed by group III, group II and group I, however no significant difference were observed. Result clearly indicated that replacement of concentrate with Kitchen waste and green berseem increased the weight gain during entire growing stage but no significant difference were observed except at 3rd fortnight where significantly highest ($P < 0.05$) weight gain was observed for group IV and no significant difference were observed among group I, II and III. The findings of the present study corroborated with the result of [8] who conducted experiment in Large White Yorkshire and desi grower pigs[3]. On the contrary[11] observed decrease in daily weight gain with increase in kitchen waste in diet of crossbred pigs. This might be due to crude protein content (8.81%) of kitchen waste used by scientists for pig is below National Research Council (NRC) recommendation required for growing pigs.

The overall average FCR during growing stage was 3.29 ± 0.12, 3.21 ± 0.22, 3.27 ± 0.35 and 3.09 ± 0.10, respectively in groups I, II, III and IV. Replacement of concentrate with kitchen waste along with green berseem decreased the FCR value. However, no significant differences were observed among the groups during whole period of experiment. Decreased FCR value in kitchen waste supplemented group indicates better utilization of nutrient. Moreover, in the present study in group I, the FCR value was found to be highest but non-significant with respect to all other treatments groups, which revealed poor nutrient utilization in the ration for weight gain. The results of the present study are well supported by[1].

Cost involved for production of 1 kg live weight for group I to IV was Rs. 44.87, 35.11, 25.11 and 14.42, respectively, which differ significantly ($P < 0.01$) with each other.

Conclusion

On the basis of findings of the present study it could be concluded that garbage can be incorporated up to 75% of total dry matter along with 0% green berseem without affecting the performance of pigs adversely. Substitution of concentrate for different level of garbage along with 10% green berseem improves the performance of animal and significantly reduced the cost of production.

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Probiotics and Prebiotics in Treatment of Diarrhea of Mink

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Summary: At present mink diseases diarrhea is considered one of the most essential urgent issues of veterinary medicine in the Latvia. The mode of action of probiotics includes maintaining normal intestinal microflora by competitive exclusion and antagonism and stimulating the immune system. The aim of the study was to determine the efficiency of probiotics with Jerusalem artichoke on the intestinal microbiota and morphology of mink with acute diarrhea.

Twenty dark brown mink of seven months of age with clinical signs of acute diarrhea were fed with basal diet and 14 days added 0.5 g *Lactobacillus reuteri* (1×10^8 CFU/g), 0.5 g *Pediococcus pentosaceus* (1×10^8 CFU/g) and 0.5 g powder of *Jerusalem artichoke* (LPJ) to each animal once a day.

It was determined that after 14 days of LPJ intake 70% animals without clinical diarrhea were observed. In non-diarrhea animals fecal samples, total microbial count, *Enterobacteriaceae*, *E. coli*, *Campylobacter coli* and *Clostridium difficile* levels were decreased by 18%, 22%, 37%, 39% and 90% ($P < 0.05$), respectively in comparision to others experimental minks. The *Lactobacillus spp.* amount after 14 days of intake significantly ($P < 0.05$) increase (32%) in mink with recovered intestine health. Histopathological changes were seen in jejunum of minks only with signs of clinical diarrhea. Supplementation of probiotics and prebiotics provides increase of gastrointestinal health of animals.

Introduction

Bacterial enteritis of mink occurs sporadically and occasionally may develop serious problems in fur farms. Traditionally treatment of animal enteritis includes using of antibiotics which can lead to formation of antibiotic resistant strains of bacteria. Therefore, use of antibiotics in treatment of enteritis of livestock is increasingly replaced by the use of probiotics and prebiotics [1], specific feed additives that favorably affect animal performance and welfare, particularly through the modulation of the gut microbiota which plays a critical role in maintaining host health [2]. The beneficial modes of action for the probiotics include: regulation of intestinal microbial homeostasis, stabilization of the gastrointestinal barrier function [3], expression of bacteriocins [4] and immunomodulatory effects [3].

Intestinal epithelial cells must coexist with a high density of diverse bacteria. Protection against these bacteria exists on multiple levels the impermeability of the intestinal epithelial barrier and serving as a protective barrier [5]. Therefore the purpose of this study was to determine the efficiency of probiotics with *Jerusalem artichoke* on the intestinal microbiota and morphology of mink with acute diarrhea.

Material and methods

Twenty dark brown mink of seven months of age (at time of pelting) with clinical signs of acute diarrhea were used. Experimental mink were fed with basal diet and 14 days added 0.5 g *Lactobacillus reuteri* (1×10^8 CFU/g), 0.5 g *Pediococcus pentosaceus* (1×10^8 CFU/g) and 0.5 g powder of *Jerusalem artichoke* (LPJ) to each animal once a day. For the evaluation of microbiological parameters, fecal samples were collected from the rectum. Total lactobacilli and *Enterobacteriaceae* counts were determined as described by van Winsen et al. [6]. For detection of *Escherichia coli* O157 Chromogenic *E. coli* Agar was used. *Campylobacter* spp. was isolated in accordance with Manual of Clinical Microbiology. All bacterial counts were expressed as $\log_{10}$ colony-forming units per gram (CFU/g).

Multiple 6 m-thick sections of the paraffin-embedded mink jejunum were examined for histology (H/E) and immunohistochemistry. The primary antibodies utilized in immunohistochemistry were polyclonal antibodies specific for serotonin (5-HT). Semi-quantitative analysis was used to estimate proportions of immunopositive cells in intestine [7]. The designations were as follows: (+) – few positive cells; (++) – moderate and (+++) – numerous positive cells in the view field.

Results and discussion

The gut microbiota, with its metabolic, trophic and protective functions, is able to affect positively the integrity of the intestinal barrier. Intestinal barrier dysfunction leads to a progressive increase of intestinal permeability, inducing a switch from "physiological" to

"pathological" inflammation that is characteristic of intestinal diseases [8]. Probiotic/prebiotic treatment positively influenced mink health. In this study, after 14 days of intake of *Lactobacillus reuteri*, *Pediococcus pentosaceus* and powder of *Jerusalem artichoke* (LPJ) 70% animals without clinical haemorrhagic diarrhea were observed. In non-diarrhea animals (50% from all minks after feeding with LPJ), total microbial count, *Enterobacteriaceae*, *E. coli*, *Campylobacter coli* and *Clostridium difficile* levels were decreased by 18%, 22%, 37%, 39% and 90%, respectively in comparision to others experimental minks. The *Lactobacillus* spp. amount after 14 days of intake significantly ($P < 0.05$) increase (32%) in mink with recovered intestine health. Other scientists [9] also observed that supplementation of the diet of neonatal pigs with a strain of *Lactobacillus* resulted in an increase in total gut populations of lactobacilli, but synbiotic product containing *L. plantarum*, maltodextrin and/or fructooligosaccharides (FOS) reduced counts of *E. coli* in the jejunum and colon of piglets [10]. Results in our study can explain with action of probiotics-adhere to the epithelial gut mucosa through binding to surface layer proteins so they do not allow free space for pathogenic microorganisms to adhere [11] and with fact that *Lactobacillus reuteri* produce reuterin-a broad-spectrum antibiotic, which is active against Gram-positive and Gram-negative bacteria, yeast, fungi, protozoa and viruses [12].

Nutritional factors and bacterial populations colonizing the gastrointestinal tract, induce functional changes in the intestinal mucosa. In the current trial, histopathological changes were seen in jejunum of minks only with signs of clinical diarrhea. Major villous changes showed fusion, full-bloodedness, destruction and loss of epithelial cells and great amount of inflammatory cells. This points to fact that neutrophilia and lymphopenia could be a response of the immune system to inflammation such as bacterial invasion. In jejunum of minks without signs of diarrhea only weak infiltration of inflammatory cells were observed that fall with decreased bacterial amount in intestine, but increased amount of goblet cells indicate to better protection and lubrication the lining of the intestine [13] in comparision with diarrhea minks.

Regulation of digestive activity by the enteric nervous system is mediated by subsets of enteroendocrine cells, which are specialized to act as sensory transducers [14]. The most well characterized of sensory transmitters is serotonin (5-HT), which is produced and secreted by enterochromaffin (EC) cells [15]. In present study, serotonin in jejunum of non-diarrhea minks was found only between muscular layers, but in animals with maintained diarrhea 5-HT expression in crypts (with basal localization) and in the middle of villi was seen, too. Results can explain with fact that the diarrhea and increased motility might be attributable to the potentiation of serotonergic signaling and causes more submucosal primary afferent neurons to respond to 5-HT-releasing mucosal stimuli [14].

Conclusion

Supplementation of diet with *Lactobacillus reuteri*, *Pediococcus pentosaceus* and powder of *Jerusalem artichoke* cause significant decrease of diarrhea of minks and provides increase of gastrointestinal health of animals.

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Effect of *Aspergillus niger and Trichoderma longibrachatium* in Degradability of Energy and Crude Protein from Alfalfa

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Summary: We evaluated a xylanase enzyme site (Fibrozyme, Alltech, Inc.) consisting of fermentation extracts and *Aspergillus niger Trichoderma longibrachatium* at different ruminal incubation periods (48, 24, 12, 8, 6, 4 and 2 h) alfalfa hay. The variables were: DNEg and CPR and nonlinear parameters. T2 (addition of enzyme) was higher in Deng from 7 to 18 h, more efficient: form 48.2% – 37.72% in total and 37.88% ($P < 0.05$). No differences ($P < 0.05$) in the mean total for CPR (crude protein residues). The CPR evident ($P < 0.01$) higher effective degradability for 20.0606^{a} *vs.* 16.3083^{b} T2 = (T1) and degradability rate 9.40^{a} h^{-1} (T2) and 0.16^{b} (T1). We conclude that adding the xylanase enzyme production improvement expectations due to its effect reflected in increased availability of net energy.

Introduction

Exogenous enzymes (Fibrozyme) in ruminant feeding affect energy intake, increased ($P < 0.05$) *in situ* degradability of NEg in 36.64% (Guerra et al., 2003). Zinn and Salinas (1999) cite an increase of 5% in digestion ($P < 0.05$) of N ration. The fiber digestion in ruminants provides the ability to convert carbohydrates into energy and protein values (Cheng et al., 1989). We evaluated the effect of in situ enzyme in these parameters Fibrozyme based rations of alfalfa hay.

Material and methods

Xylanase enzyme was evaluated (Fibrozyme, Alltech, Inc.) consisting of fermentation extracts and Aspergillus niger Trichoderma longibrachatium in two treatments T1 = alfalfa, alfalfa more Fibrozyme = T2. Basal diet: alfalfa hay *ad libitum* and 3 kg of concentrate d^{-1} (Fodder El Barrio), for ten days. The enzyme was dosed: 14 $g \cdot d^{-1}$, 7 ga 7 ga 0800 and 2000 h, two male animals holstein-gyr 3/4 cannulated in rumen weight of 800 kg. Introduced per animal per treatment 112 nylon bags 10 cm × 20 cm (ANKOM) with 5 g of ground sample (1 mm) of alfalfa, weighed and identified by bag, animal and period, with a pore size of 50 ± 15 μm, and exhibition area of 18 $cm^2 \cdot mg^{-1}$. We estimated CP, DM (AOAC, 1975) and NEg (Zinn and Salinas, 1999) Residues of crude protein (CPR) and degradability of NEg (NEgD) was evaluated at different incubation periods 48, 24, 12, 8, 6, 4 and 2 h. After incubation the pouches were washed with running water for 5 min, to be clean. For *in situ* degradability formula was used Flatt and Schneider (1975). Nonlinear parameters were estimated rumen (NLPR) RPC (Ørskov and McDonald, 1979) with the exponential equation: $p = a + b(e^{-ct})$, where: p = rate of disappearance of nutrients in a while, a = intercept of the solubilized portion at the beginning of the incubation (time 0), b = potentially degradable fraction in the rumen, c = speed or rate of degradability of fraction b, and t = time of incubation. Potential degradability was obtained (dp) = a + by effective degradability (ed) = a + b * c/(c + 0003) assuming a passage rate of 3% (Ørskov and McDonald, 1979). DBCA was employed, proof χ^2 and Tukey mean comparison (Barreras et al., 1997). Statistical analyzes were performed using the SAS statistical package (2001).

Results and discussion

The NEgD was different ($P < 0.05$) for treatment periods. T2 showed superiority from 7 to 18 h, more efficiently: 48.2% – 37.72% (Fig. 1). The total undifferentiated NEgD periods shows that T2 was higher ($P < 0.05$) to 37.88% in T1 (T1 = 0.57889^{b} *vs.* T2 = 0.79821^{a} Mcal · kg^{-1} DM). These results agree with Guerra et al. (2003) who evaluated the *in situ* degradability of Fibrozyme finding ($P < 0.05$) increased at 48 h NEg of 36.64%. Fibrozyme enzyme affects the energy input. Crude protein residues (CPR) differed ($P < 0.05$) when only 6.24 and 48 for the $period^{-1}$ treatments. T2 times in 6, 24 and 48 indicates that it is more efficient ($P < 0.05$) in the range of 2.02% to 9.30% (Fig. 2), no differences ($P > 0.05$) in the overall

mean comparison(T1 = 18. 79[a] *vs*. 18. 47[a]% = T2) .

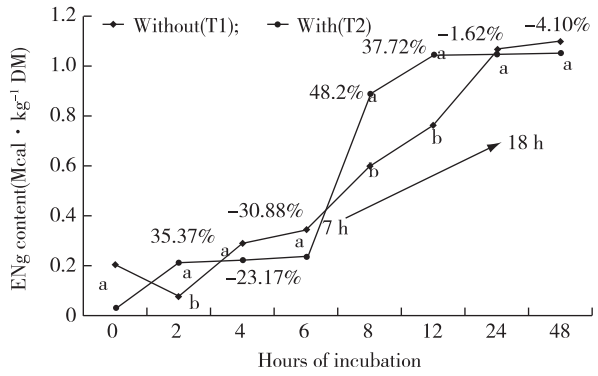

Fig. 1 NEg Degradability h $^{-1}$
Values with different literal differ statistically (P < 0. 05)
Tukey test (C. V. = 2. 21 – 18. 0)

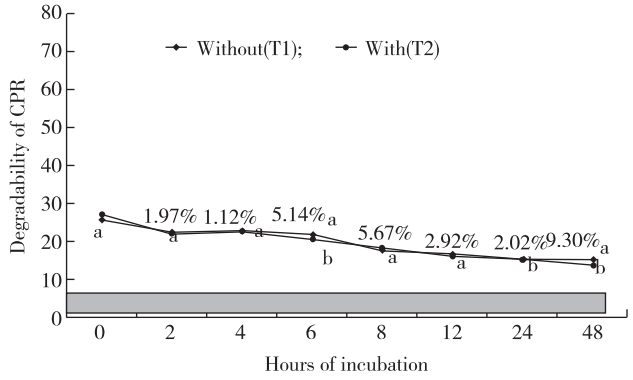

Fig. 2 Waste Crude Protein% h $^{-1}$
Values with different literal differ statistically (P < 0. 05)
Tukey test (C. V. = 1. 66 – 8. 75)

The CPR NLPR evident(P < 0. 01) higher effective degradability for T2 with 20. 0606[a] vs. 16. 3083[b] and a rate of degradability 9. 40[a] h $^{-1}$ (T2) and 0. 16[b] (T1) (Table 1). Although there was no(P > 0. 05) differences between the mean total of CPR, the addition of the enzyme improved(P < 0. 05) degradability rate in effect on the effective degradability.

Table 1 Nonlinear parameters *In situ* ruminal degradability for CP

Parameters	CP	
	T1(without)	**T2(with)**
a	23. 891[a]	24. 757[a]
b	11. 142[a]	11. 1416[a]
c(h $^{-1}$)	0. 0016[b]	0. 094[a]
dp(a + b)	35. 033[a]	35. 89[a]
ed	16. 3083[b]	20. 0606[a]

Values in the same row with different literal differ statistically (P < 0. 01) χ^2

Conclusions

These results demonstrate that the addition of exogenous enzymes in feed for ruminants increase the contribution of NEg in significantly(37. 88%) , but does not affect the CP wastes about different incubation periods. We conclude that adding Fibrozyme improved production expectations due to its effect reflected in increased availability of net energy gain between 7 and 18 hours after ingestion of food.

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Litter Types and Health of Foot Pads in Broilers and Fattening Turkeys Fed Identical Diets

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Summary: Foot pad dermatitis (FPD) is a widespread challenge affecting poultry health and welfare. In the field different types are used, therefore there is a need to compare among those litter materials in both broilers and fattening turkeys regarding foot pad health. Four trials were done at the university research farm. In each trial identical diets were fed to all groups. Trial 1:18500 broiler (Ross 308) were divided into 2 groups, housed either on wood shavings or straw-granulate (1000 g/m^2). Trial 2: Two trials were done, 19800/18500 broilers (in trials 1/2) were divided into 2 groups. Birds were housed either with wood shavings or lignocellouse (Soft Cell®) (1000 g/m^2). Trial 3: 3092 turkeys were divided into 2 groups, housed either on wood shavings (7.6 kg/m^2) or lignocellouse (7 kg/m^2) till the first 5 weeks, then both groups were housed only on wood shavings. Trial 4: 3134 turkeys were divided in to 2 groups, housed either on wood shavings (7.6 kg/m^2) or straw-granulate (4 kg/m^2) till the first 5 weeks, then both groups were housed only on wood shavings. At end of each trial, 200 foot pads/group were examined for FPD scores. The results shows that in Trial 1: Birds housed on wood shavings were accompanied with significant higher FPD scores (4.32 ± 1.60) vs. (3.27 ± 1.67) for straw-granulate. Trial 2: Lignocellose resulted in significant lower FPD scores (2.81 ± 1.72 and 3.15 ± 1.59 in trials 1 and 2) vs. (3.60 ± 1.59 and 4.05 ± 1.48 in trials 1 and 2) for wood shavings. Trial 3: No significant differences were found between wood shavings and lignocellouse regarding health of foot pads (3.96 ± 0.9 and 3.80 ± 1.0, respectively).

Trial 4: Wood shavings were associated with significant lower FPD scores (3.80 ± 0.96) in comparison to straw-granulate (4.04 ± 1.1). It is concluded that types of litter material markedly affect the health of foot pads inspite of identical diets.

Introduction

The incidence and severity of foot pad dermatitis (FPD) is of great concern not only to the poultry industry but also the welfare of the birds. The FPD lesions range from discolouration at an early stage, then hyperkeratosis and necrosis of the epidermis to ulcers in severe cases [3]. Many factors have been implicated in the prevalence of FPD. Nevertheless, different authors have found positive correlations between good litter quality, particularly low moisture, and the incidence of FPD [4]. Nutrition is considered to be a major factor in the onset of FPD along with poor litter quality. A high dietary level of soybean meal (SBM) is thought to be one of the nutritional factors causing FPD. It is well known that SBM is the most common protein source for use in turkey diets. The protein requirements of turkeys are high, especially in early stages of life, thus high proportions of SBM in their diets being included. However, standing on wet litter brings the feet in constant contact with moisture and has been suggested to cause the foot pad to soften and become more prone to damage, predisposing the bird to developing FPD [3, 6]. The first marked increase of FPD lesion was observed after exposure for 4 h/d to 35% moisture which was nominated as "critical moisture content" [2]. Birds are mostly in close contact with litter during their life. Therefore, type and quality of litter are of special interest in the incidence of FPD. The effects of litter material on FPD are thought to be due either to the physical structure (hard or soft) or different water binding capacity (higher or lower) of the litter. The most common bedding materials used for poultry (broilers or turkeys) are wood shavings and/or cereal straw [8, 1], but there are currently further litter types which can be also used as lignocellulose (Soft Cell®) and straw granulate pellets. Therefore there is a need to compare among those litter materials in both broilers and fattening turkeys regarding health of foot pads.

Material and methods

Four trials were done at the university research farm. In each trial identical diets were fed to all groups. Trial 1: 18500 broiler (Ross 308) were divided into 2 groups, housed either on wood shavings or straw-granulate (1000 g/m^2). Trial 2: Two trials were done, 19800/18500 broilers (in trials 1/2) were divided into 2 groups. Birds were housed either with wood shavings or lignocellouse (1000 g/m^2). Trial 3: 3092 turkeys (BUT Big 6) were divided into 2 groups, housed either on wood shavings (7.6 kg/m^2) or lignocellouse (7 kg/m^2) till the first 5

weeks, then both groups were housed only on wood shavings. Trial 4: 3134 turkeys (BUT Big 6) were divided in to 2 groups, housed either on wood shavings (7.6 kg/m^2) or straw-granulate (4 kg/m^2) till the first 5 weeks, then both groups were housed only on wood shavings. All birds being allocated to a floor pen (472 m^2/group). Feed and water were available *ad libitum* for all groups. All were housed under identical husbandry conditions and going through a normal fattening procedure (6 phases for turkeys and 3 phases for broilers). At the end of fattening period in each trial, the foot pads were assessed (200 foot pads/group). Only the central plantar area was scored, signs of foot pad lesions were assessed on a 7-point scale (0 = normal skin; 7 = over half of foot pad is covered with necrotic scales) according to MAYNE et al. [6].

Results

Table 1 shows that in Trial 1: Birds housed on wood shavings were accompanied with significant higher FPD scores (4.32 ± 1.60) vs. (3.27 ± 1.67) for straw-granulate. Moreover, It was noted that the mean of the dry matter (DM) content of litter throughout the experimental period was markedly higher for straw-granulate (78.6% DM) compared to wood shavings (72.9%). Trial 2: Lignocellose resulted in significant lower FPD scores (2.81 ± 1.72 and 3.15 ± 1.59 in trials 1 and 2) vs. (3.60 ± 1.59 and 4.05 ± 1.48 in trials 1 and 2) for wood shavings (Table 2). Trial 3 as shown in Table 3: No significant differences were found between wood shavings and lignocellouse regarding health of foot pads (3.96 ± 0.9 and 3.80 ± 1.0, respectively). Trial 4: Wood shavings were associated with significant lower FPD scores (3.80 ± 0.96) in comparison to straw-granulate (4.04 ± 1.1) as shown in Table 4.

Table 1 Effects of different litter materials on health of foot pads in broilers (Trial 1)
According to RADKO et al. [7].

Day of life	FPD Scores	Wood shavings			Straw-granulate		
		0 – 3.5	4 – 5.5	6 – 7	0 – 3.5	4 – 5.5	6 – 7
11	mean		0.61^a ± 1.0			0.18^b ± 0.4	
	n = 150 birds	146	4	0	150	0	0
23	mean		2.72^a ± 1.8			1.71^b ± 1.2	
	n = 150 birds	105	30	15	138	9	3
35	mean		4.32^a ± 1.6			3.27^b ± 1.6	
	n = 200 foots	39	117	44	87	96	17

Table 2 Effects of different litter materials on health of foot pads in broilers (Trial 2)
According to KAMPHUES et al. [5].

	Wood shavings	Lignocellulose
Trial 1		
examined foot pads, n	200	200
FPD scores, mean	3.60^a ± 1.59	2.81^b ± 1.72
prevalence of birds, %		
score = 4 – 5	57.0	42.5
score = 6 – 7	8.50	4.50
trial 2		
examined foot pads, n	200	200
FPD scores, mean	4.05^a ± 1.48	3.15^b ± 1.59
prevalence of birds, %		
score = 4 – 5	73.5	53.0
score = 6 – 7	10.0	2.50

Table 3 Effects of different litter materials on health of foot pads in turkeys (Trial 3)

	Wood shavings	Lignocellulose
First 5 wk of life		
examined of birds, n	150	150
FPD scores, mean	4.20^a ± 0.6	3.20^b ± 0.80
prevalence of birds, %		
score = 0 – 3.5	91	121
score = 4 – 5.5	59	29
At end of fattening		
examined foot pads, n	200	200
FPD scores, mean	3.96^a ± 0.9	3.80^a ± 1.0
prevalence of birds, %		
score = 4 – 5	69	64.5
score = 6 – 7	5.5	4.5

Discussion

Poultry spend most of their productive life in close contact with the litter material and hence the type of litter appears to have a marked effect on the incidence of FPD [1]. Of all bedding materials tested, lignocellulose showed the lowest severity of FPD in broilers with a very low prevalence of scores 6 – 7. This could be related to its higher water absorbing capacity and also to the rapid release of water. These results are consistent with those

found in recent studies [8,1]. However, even with using lignocellulose for only fist 5 wk of life in turkeys negative effects were observed at end of fattening period.

Table 4 Effects of different litter materials on health of foot pads in turkeys(Trial 4)

	wood shavings	straw-granulate
First 5 wk of life		
examined of birds, n	150	150
FPD scores, mean	$4.24^{b} \pm 0.48$	$4.35^{a} \pm 0.47$
prevalence of birds,%		
score = 0 – 3.5	10	4
score = 4 – 5.5	138	145
At end of fattening		
examined foot pads, n	200	200
FPD scores, mean	$3.80^{b} \pm 0.96$	$4.04^{a} \pm 1.1$
prevalence of birds,%		
score = 4 – 5	70.5	70.0
score = 6 – 7	1.50	16.0

Interestingly, the straw-granulate pellets in broilers had a positive effects regarding the severity of foot pads but not in fattening turkeys. It could be due to differences in the physical/chemical structure of both of them. It is believed that the effects of litter material on FPD are thought to be due to either the physical structure(hard or soft) or the water-binding capacity(high or low) of the litter [8,1]. Litter must no only be able to absorb moisture but should also have a reasonable drying time to get rid of that moisture via evaporation.

Conclusions

The development and severity of FPD varied significantly among the bedding materials and correlated substantially to the moisture content in the litter. The present results indicate that lignocellulose could reduce the severity of FPD either in broilers or in turkeys, probably due to the higher binding capacity and also quick release of water(higher evaporation). However, lignocellulose will never be used for the whole fattening period(20 wks) in turkeys, due to its high costs(12.5 kg/m^2 = 5 €/m^2). Straw-granulate seemed to be a desirable litter material for broilers but not for turkeys.

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Providing Supplemental Milk during Lactation: Effects on Sows' and Piglets' Health and Performance

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Summary: One way to handle large litter sizes and to raise as many piglets as possible is to offer ad libitum supplemental milk in addition to sow's milk in the farrowing crate. One aspect of this management practice is the reduction of cross fostering, which contributes to an increased level of animal hygiene. Another aspect is the improvement of animal health and welfare. The aim of this study was to investigate the influence of supplemental milk on health and performance of the sow and her litter. In the supplemented group(SUPP), piglets had free access to the supplemental milk, provided by special cups, from the 2nd day of life on. Both groups, SUPP and the control group(CONT), received Prestarter from the 7th day of life. Because of animal welfare aspects and the experimental design SUPP sows retained as many piglets as they had functional teats, whereas CONT sows retained one piglet less than they had functional teats.

In SUPP, 13.5 pigs, and in CONT, 12.4 pigs per sow were weaned after 27 suckling days. The total litter weight was higher in the SUPP group(SUPP: 104.9 kg; CONT: 96.7 kg; $P < 0.05$). The average piglet weight was similar in both groups(SUPP/CONT: 7.8 kg; $P > 0.05$). No significant difference was noted between piglets of CONT and SUPP in terms of piglet mortality and occurrence of diarrhea. With respect to the sow, no significant differences were detected for the body-condition-score, the body weight and the backfat thickness between the groups in the period between housing in and out. Via clinical examination, no significant difference was assessed regarding the occurrence of Postpartum Dysgalactia Syndrome in the first three days of life in both groups(SUPP: 8.3%; CONT: 5.0%; $P = 0.7$). Ten sows of SUPP, and 14 sows of CONT had mastitis during a later time point of lactation.

Introduction

Over the last decades, the number of piglets born alive per litter has increased rapidly. Therefore, the demands on a proper management in the farrowing are higher than ever before. High-prolific litters are associated with increasing negative effects on the growth of the piglets [1], and a higher mortality rate [1,2]. To limit the number of piglets at one sow, and for adjustment of individual piglet weights, different cross fostering techniques are used. This leads to a reduced standard of animal hygiene and welfare. By offering piglets supplemental milk (Supp-Le-Milk®) in addition to the sow's milk, the current situation in farrowing units should be improved. Via this system, piglets have free access to additional intakes of milk, provided by special cups, from the 2nd day of life on.

Material and methods

The use of the technology was analyzed in the research center Futterkamp of the Chamber of Agriculture Schleswig-Holstein, where sows were kept in farrowing crates and managed with a 28 day lactation period. Supplemented sows (SUPP, n = 60 sows) and control sows(CONT; n = 60 sows) with their litters were tested between July 2011 and April 2012. Sows of the high-prolific Porkuss® genetics were used, and litters were crossbreedings with Pi train boars. Sows were randomly assigned to SUPP or CONT, and were averagely in their fourth parity. Because of animal welfare aspects, the experimental design required a balancing of the number of piglets per sow within 48 h postpartum(pp) as following: SUPP sows retained as many piglets as they had functional teats, whereas CONT sows retained one piglet less than they had functional teats.

The Supp-Le-Milk® system was installed for the trial. Starting on day two post partum(pp), SUPP piglets had ad libitum access to a milk replacer, which was prepared freshly daily. Piglets of SUPP and CONT received Prestarter from the 7th day of life on.

Several parameters and measurements of SUPP and CONT were recorded(Table 1).

Table 1 Measurements of SUPP and CONT

Sows	Piglets
Investigation [time points]	**Investigation [time points]**
number of piglets [at time of birth and weaning] body-condition-score [housing in and out] body weight [housing in and out] backfat thickness via ultrasound technique [weekly] clinical examination(e.g. mammary gland) [daily] microbiological analyses of sow's milk samples (CONT: n=116, SUPP: n=116) [at day 2, 14 and 20 of lactation]	body weight [after birth, at day 7, 14 and at time of weaning] losses of piglets [suckling period] diarrhoea occurrence (severity graded into categories: 0 = no occurrence, 1 = slight, 2 = intermediate, 3 = severe) [daily]

Data were analyzed using SAS-software (SAS 9.2, Institute Inc., Cary, NC, USA). Body weight of sows and piglets, number of piglets, backfat thickness and body-condition-score of sows were examined using a generalized linear mixed model (Mixed-procedure) involving fixed effects (group, batch, parity number), random effects (sow), and covariates (duration of suckling period, except for backfat thickness). The results of these analyses are expressed as least squares means (LSM). With regard to clinical examinations, microbiological analyses and mortality, differences between CONT and SUPP were tested with Chi -test (Freq-procedure). Results with $P < 0.05$ were regarded as significant.

Results

In SUPP, 13.5 piglets, and in CONT, 12.4 piglets were weaned. At time of weaning, piglets weighed 7.8 kg both in CONT and SUPP. A difference became apparent by the total weaning weight of the litter in SUPP, which was significantly higher than in CONT (CONT = 96.7 kg; SUPP = 104.9 kg).

Piglet mortality occurred on average at day 2.6 pp in CONT, and at day 2.4 in SUPP. Piglet mortality in CONT (16.4%) was higher than in SUPP (13.8%) in tendency ($P = 0.1$). The occurrence of diarrhea reached no statistically significant differences. Piglets of CONT showed an average grade of 0.1, and piglets of SUPP were assessed with an average of 0.2.

In this study, SUPP sows lost more body weight than CONT sows in tendency ($P = 0.4$) (Table 2). For sows of both groups, an equal decrease of body condition score was observed between housing in and out. Furthermore, the loss of backfat thickness was not significantly different, but had an unexpected higher starting value in SUPP (Fig. 1). Both groups did not differ with regard to their feed uptake.

Table 2 Least squares means (LSM) and standard error (SE) of body weight and body-condition score of sows at the time of housing in and housing out

	Body weight in kg				Body-condition-score			
	CONT		SUPP		CONT		SUPP	
	LSM	SE	LSM	SE	LSM	SE	LSM	SE
Housing in	271.2[a]	2.2	271.9[a]	2.1	3.8[a]	0.1	3.9[a]	0.1
Housing out	236.5[a]	2.8	234.2[a]	2.8	2.9[a]	0.1	2.9[a]	0.1

[a, b] different letters indicate significant differences ($\alpha = 5\%$).

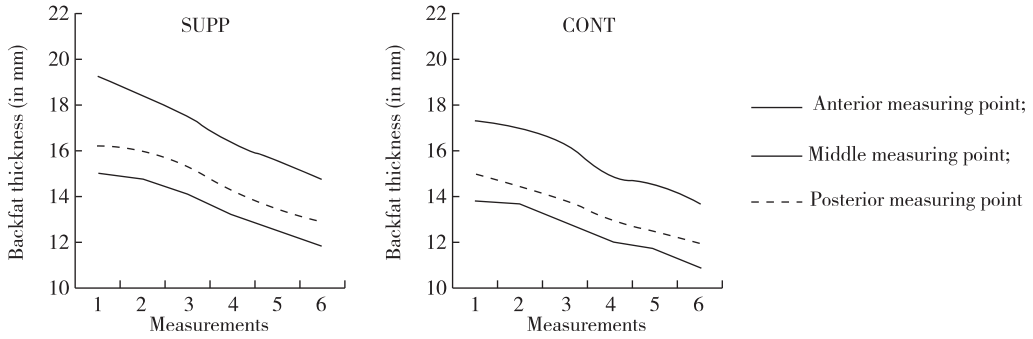

Fig. 1 Loss of backfat thickness during lactation (in mm)

The clinical examination for mastitis showed that three sows of CONT and five sows of SUPP became affected by the Postpartum Dysgalactia Syndrome within 3 days pp. Under consideration of advanced lactation, ten sows of SUPP and 14 sows of CONT showed mastitis.

Microbiological analysis did not show any statistically significant difference in the occurrence of bacteria in sow's milk between CONT and SUPP. The mainly isolated species belong to the families *Staphylococcaceae*, *Streptococcaceae*, and *Enterobacteriaceae*.

Discussion

In response to increasing litter sizes, average growth rates of individual piglets decreases [3]. This study showed that with an additional offer of supplemental milk, individual weaning weights of CONT and SUPP are equal, despite the fact that in the SUPP group, one more piglet had to be fed. Contrary to other recent studies [4 – 6], no significant rise in weaning weights was observed by the provision of supplemental milk. However, in these trials sows fostered the same number of piglets in CONT and SUPP in contrast to the current study.

There was no significant impact of milk replacer on survival rates of piglets, as it has been already described in literature [4]. One possible interpretation is that piglets have access to milk replacer at day two of their life after sufficient colostrum consumption. However, in a recent study KilBride et al. [7] found that 62% of preweaning deaths occurred in the first two days. The occurrence of diarrhea was slightly, but not statistically significant, increased in SUPP.

The body weight of sows decreased with increasing litter size [8,9]. Also backfat loss increased [10]. In this study, the loss of body weight during lactation in SUPP tended to be a little more than in CONT, but without significant differences. The reason for slightly higher losses of body weight could be that sows of SUPP, who consumed an equal quantity of food as CONT, had to raise one more piglet than CONT sows. By feeding their piglets supplemental milk, a better stimulation of milk production of the sow can be expected [11], which in turn leads to mobilizing body tissue. However, the loss of backfat thickness and the body-condition-score between the groups in the period between housing in and out were similar (Table 1). The high incidence of *Staphylococcaceae* and *Streptococcacea* was also reported by Kemper and Preissler [12], who examined colostrum before a piglet suckled.

Conclusions

Supplemental milk supports fostering large litters and makes piglet more independent from the milk production limits of sows. Animal hygiene, health and welfare are improved, and cross fostering is reduced.

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Experimental Studies on Toxic Interaction of Oxytetracycline and Cadmium

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Summary: These experimental studies were carried out on 450 Ross chicks divided into 6 groups. Some of these groups administered a therapeutic dose of oxytetracycline(OTC) plus cadmium(0.1 ppm cadmium chloride in drinking water), other groups were given over concentration of OTC(6 g/l of drinking water), double the therapeutic dose plus the same dose of cadmium chloride. It had been reported that administration of OTC, OTC plus cadmium had a serious effect on both the muscular tissue and the parynchymatous organs including hepatocellular degeneration, focal lymphoid cell reaction and fibroblastic reaction in the portal area of liver, nephrosis of kidney with degeneration of some renal tubules and depletion of lymphoid cells of white pulp of spleen. It was also associated with cellular immune suppression. So new laws regulating administration of antibiotic should be enforced.
Key words: oxytetracycline, cadmium, toxicity, chicks

Introduction

Oxytetracycline (OTC), tetracycline, chlortetracycline, and doxycycline, have for decades continued to play an important role in veterinary medicine and feed additives because of their broad spectrum activity and economical advantages[1]. Cadmium has been shown to suppress antibody formation in animals and has been epidemiologically linked to respiratory cancer[2]. One of the stress factors is cadmium content in water supplies or feed stuffs. Cadmium is a toxic transition metal of continuing occupational and environmental concern with a wide variety of adverse effects and has an extremely long biological half-life 20 – 30 years that essentially makes it accumulative toxicant in human. It has been also shown to have effects on a variety of tissues and biological system and has been associated with such diverse maladies as hypertension and carcinogenesis. In several epidemiological studies cadmium exposure in the work place has been linked to carcinogenesis in various tissues including the lung, prostate, kidney and stomach[3, 4]. It has been stated that cadmium induced renal lesions that are not reversible, which may interfere with OTC clearance leading to high residual levels in chicken tissues. The present study was designed to evaluate toxic effects of OTC on broiler chickens due to therapeutic dose and overdose at assiut environment. And evaluate toxic effect of OTC under previous conditions associated with stress of environmental pollutants in water as cadmium.

Material and methods

450 Ross chicks of both sexes at age of one day old were obtained from Assiut National Company of poultry and eggs. Oxyteteracycline hydrochloride powder (100%) concentration, water soluble was obtained from (Pharco Company, Egypt). And use Cadmium chloride (Merck). Birds were divided into two groups (A and B), each contain(225) broiler chickens.

a. Group(A):

Birds were fed on OTC free ration and drinking tap water from day one till the end of experiment. After 31 days age, these birds were divided into three sub-groups (A1, A2 and A3) each contained 72 birds and treated as follow:

1 – Sub-group A1: This group was left as control till the end of the experiment.

2 – Sub-group A2: This group was given therapeutic dose(3 g OTC/L tap water) for 5 successive days.

3 – Sub-group A3: This group was given overdose of OTC (6 g OTC/L Double therapeutic dose) for 5 successive days.

b. Group(B):

as group A in addition to 0.1 ppm cadmium chloride, which added to drinking water from day one till the end of experiment. These birds were also divided into three sub-groups (B1, B2 and B3) after 31 days age; each contains 72 chicks and treated as follow:

1 – Sub-group B1: This group was kept as control for group B.

2 – Sub-group B2: This group was given OTC therapeutic dose(3 g OTC/L tap water) for 5 successive days in drinking water.

3 – Sup-group B3: This group was given over dose of OTC for (6 g OTC/L Double therapeutic dose) 5 successive days in drinking water.

After stopping of OTC administration 6 chickens from

all groups (A1, A2, A3, B1, B2 and B3) were slaughterd day after day at age of 37th days till the end of experiment and samples from liver, kidneys, spleen, bursa of fabricius and bone samples were obtained.

Results

The histopathological changes of the investigated organs of broiler chickens (liver, kidney, spleen and bursa of fabricius) revealed pronounced, moderate to mild changes or appeared more or less normal depending upon the various handled groups of the experiment and control. The liver showed slight to mild hyperemia, degeneration, portal fibrosis and Kuppfer cells activation. Focal areas of hepatic necrosis and lymphoid cell reaction at portal tract in others. The liver of chickens from group B1 showed mild hyperemia, degenerative changes included vaculation of liver cells and mild portal fibrosis. The kidney showed degenerative and necrotic changes in the tubular epithelium (nephrosis), some areas of Hemorrhage, mild increase in cellularity of the glomerular tuft and focal areas of interstitial reaction. Spleen showed severe depletion of lymphoid cell population of the white pulp which is replaced by proliferation of the reticuloendothelial system cells. Prominent depletion of lymphoid cells population in the bursa of Fabricius.

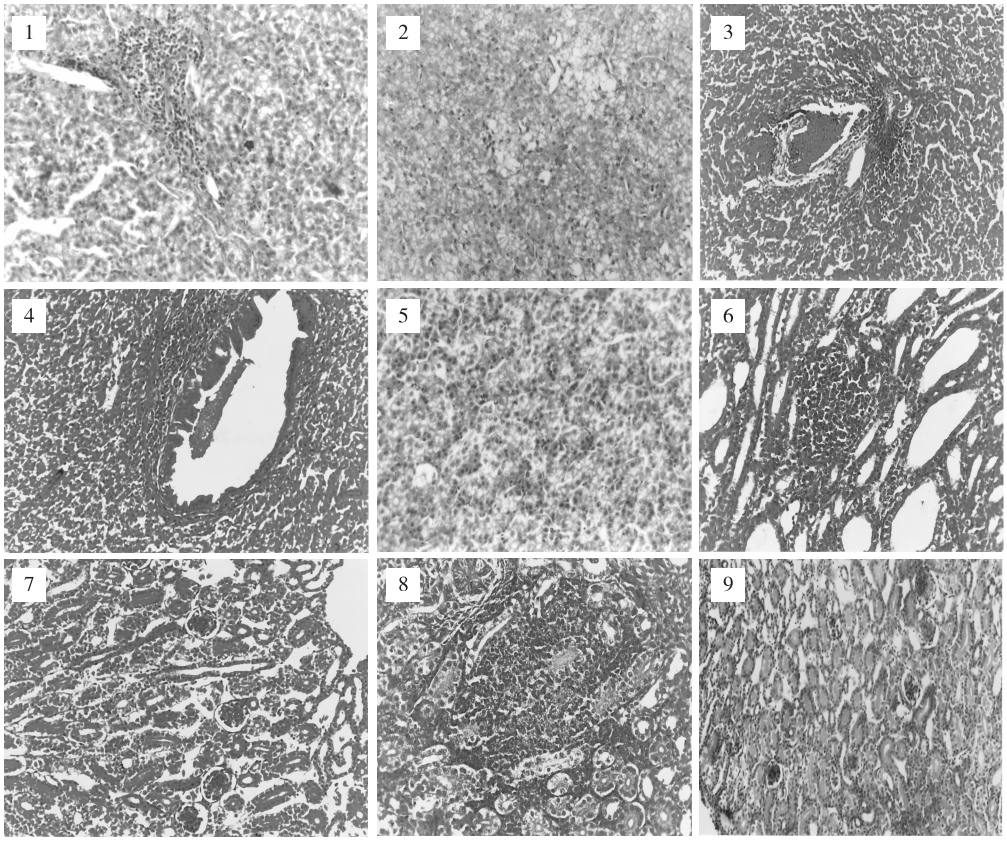

Fig. 1 Liver showing focal area of hepatic necrosis and lymphoid cell reaction at portal tract. Group A2, stain H & E, (×25).
Fig. 2 Liver showing small multiple area of degeneration. Group A3, stain H & E, (×25).
Fig. 3 Liver showing thrombosis and cellular reaction in the blood vessels of the portal tract. Group A3, stain H & E, (×25).
Fig. 4 Liver showing hyperplasia of the bile duct epithelium. Group B3, stain H & E, (×25).
Fig. 5 Liver showing degeneration, vaculation of hepatic cell cytoplasm. Group A3, , (×25).
Fig. 6 Kidney showing cystic dilation of the renal tubules with atrophy of their epithelium and some dilated tubules showed complete loss of its epithelium. Focal area of lymphoid cell reaction. Group A2, stain H & E, (×25).
Fig. 7 Kidney showing necrosis of glomerular tuft and degenerative changes of renal tubular epithelium. Group A2, stain H & E, (×25).
Fig. 8 Kidney showing necrosis and degeneration of renal tubular epithelium and area of interstitial reaction. Group A3, stain H & E, (×25).
Fig. 9 Kidney showing necrosis of tubular epithelium and glomeruli. Group A3, stain H & E.

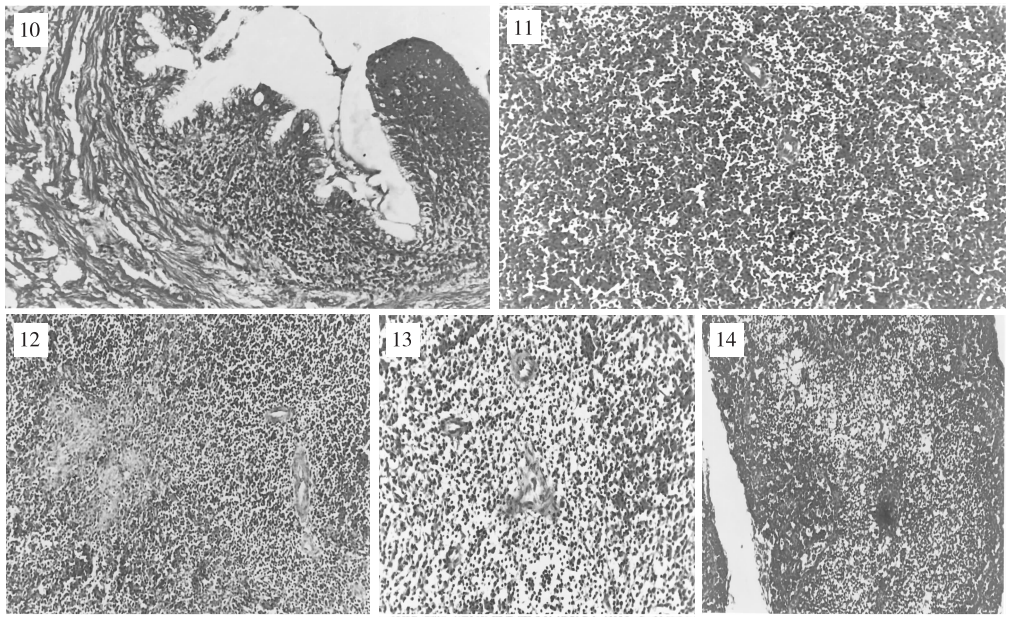

Fig. 10 Kidney showing inflammation of renal part of the ureter with increased number of goblet cells. Group A3, stain H & E, (×25).
Fig. 11 Spleen showing very mild depletion of lymphoid cell population in the white pulp and congestion of red pulp. Group A2, stain H & E, (×25).
Fig. 12 Spleen showing moderate decrease in the lymphoid cell population of the white pulp. Group A3, stain H& E, (×25).
Fig. 13 Spleen showing necrosis and depletion of lymphoid cell population. Group B3, H & E, (×25).
Fig. 14 Bursa of fabricius showing necroting changes with mild depletion of lymphoid cell population. Group A3, stain H & E, (×25).

Discussion

The OTC administrations as well as cadmium intake in water were associated with some histopathological changes in examined organs including hepatocellular degeneration of liver, nephrosis with degeneration of some renal tubules and presence of proteinous material and hyaline casts in the lumen of the tubules which indicate an increased glomerular permeability of the nephron, resulted from nephritic syndrome[5]. Adminstration of cadmium plus therapeutic or overdose of OTC resulted in severe exhaustion of lymphoid cell population in the spleen and bursa of fabricius, this was associated with immunosuppression[6].

Conclusions

We concluded that administration of OTC, OTC plus cadmium had a serious effect on both the muscular tissue and the parynchymatous organs. It was also associated with cellular immune suppression. Protection of public health from animal products which contain high levels of animal drugs residues should be adopted. So new laws regulating administration of antibiotic should be enforced and the cadmium level in water should be monitored regulary in poultry farms.

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Toxoplasma gondii: Efficacy of an Irradiated Vaccine Against Experimental Infection Challenge in Wistar Female Rats

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Summary: Toxoplasmosis is a worldwide zoonotic disease caused by *Toxoplasma gondii* (*T. gondii*) protozoa. It causes economic losses in livestock production and also serious implications on Public Health, due severe neurological and reproductive symptoms. Prevention measures for *T. gondii* infection's block have not been reached in order to avoid toxoplasmosis occurrence. The development of anti-*T. gondii* vaccines may be an important alternative for the disease control. The objective of the present study was to evaluate the efficacy of a *T. gondii* tachizoites irradiated vaccine, administered in experimentally inoculated Wistar female rats, aiming to block the production of *T. gondii* cysts in brain and muscle tissues, and also the congenital transmission. It was verified that the immunization with irradiated tachyzoites of *T. gondii* induced the reduction of parasitic load in most organs analyzed, although not prevent the establishment of infection with the parasite. And also, the immunization showed a favorable effect on the birth rate and litters' size.

Introduction

Toxoplasmosis is a parasitic disease with high importance in Veterinary Medicine, Animal Science and Public Health contexts. It causes economic losses in farms, due reproductive fails, and also severe implications on Public Health, considering that the consumption of milk and meat form infected animals may contribute for the zoonotic transmission [1,2].

In Veterinary Medicine, the vaccines development is necessary to control the acute infection by *Toxoplasma gondii* (*T. gondii*); to prevent the congenital toxoplasmosis and to block the spread of oocystes by cats, which are the definitive hosts of this disease [3]. In livestock animals, the vaccine would be an important tool to prevent abortion; to reduce the economic losses and also to reduce or block the production of tissues cysts in dairy and beef animals, contributing to reduce the main important epidemiological chain for human infection [4]. By this way, the development of vaccines viewing to prevent or reduce the production of tissue cysts in livestock animals may be very useful in the risk reduction of the infection transmission to human [5].

In Public Health the formulation of vaccines against *T. gondii* has been intensively studied, especially focused on pregnant women and immunosuppressed patients [6, 7]. Despite several studies for antigen development have being conducted, no vaccine was approved for human use until this moment [8].

The objective of the present study was to evaluate the efficacy of a *T. gondii* tachizoites irradiated vaccine, administered in experimentally inoculated Wistar female rats, for blocking the production of *T. gondii* cysts in brain and muscle tissues, and also the congenital transmission.

Material and methods

Vaccine was composed by 1×10^7 parasites/dose, plus aluminum hydroxyl as adjuvant, irradiated at 255 Gy. Immunization was done by gavage (oral route), with three doses of 15 days interval. One week after pregnancy confirmation, challenge was done by gavage with *T. gondii* cysts, oocysts and tachizoites. Forty-eight pregnant Wistar rats were distributed in groups, composed by eight animals, as described on Table 1.

Table 1 Group components and respective treatments for the efficacy evaluation of an irradiated vaccine against *Toxoplasma gondii* experimental infection challenge in Wistar female rats. Botucatu-SP, Brazil, 2012

Group number	Vaccinated	Challenged with	Strain
1	×	1×10^2 bradizoites	ME-49 *T. gondii*
2	–	1×10^2 bradizoites	ME-49 *T. gondii*
3	×	1×10^2 oocysts	ME-49 *T. gondii*
4	–	1×10^2 oocysts	ME-49 *T. gondii*
5	×	1×10^2 tachizoites	RH *T. gondii*
6	–	1×10^2 tachizoites	RH *T. gondii*
7	×	–	–
8	–	–	–

Each group was composed by six animals.

Three weeks after infection, rats were euthanized. Tissues and organs were collected for parasite load evaluation by Real Time PCR. Neonates were euthanized soon after birth and its brains were collected for parasite load evaluation by Real Time PCR.

Results and discussion

Detailed results regarding parasitary load; fecundity levels; cysts counts, and cellular response in small intestine in the different groups are presented on Tables 2 to 5.

Table 2 Statistical analysis, median and percentage of 25% and of 75% of parasitary load by qPCR for *T. gondii* between different evaluated groups, according to the examined tissue. Botucatu-SP, Brazil, 2012

	Groups								
	1	2	3	4	5	6	7	8	P
Brain	0.0 [0.0 – 10.7] ^{B}	152.9 [0.0 – 532.5] ^{AB}	64.0 [0.0 – 496.0] ^{AB}	3212.3 [1683.0 – 6310.7] ^{A}	0.0 [0.0 – 0.0] ^{B}	0.0 [0.0 – 0.2] ^{AB}	0.0 [0.0 – 0.2] ^{AB}	0.0 [0.0 – 0.0] ^{B}	0.004
Heart	1.2 [0.0 – 10.4] ^{AB}	0.2 [0.0 – 73.4] ^{AB}	43.6 [0.5 – 161.0] ^{AB}	209.8 [69.8 – 248.8] ^{A}	0.4 [0.0 – 4.0] ^{AB}	0.7 [0.0 – 3.3] ^{AB}	1.1 [0.0 – 1.4] ^{AB}	0.0 [0.0 – 0.0] ^{B}	0.005
Spleen	0.0 [0.0 – 0.0] ^{A}	0.0 [0.0 – 0.0] ^{A}	0.0 [0.0 – 0.0] ^{A}	0.0 [0.0 – 0.0] ^{A}	0.0 [0.0 – 0.0] ^{A}	0.0 [0.0 – 0.0] ^{A}	0.0 [0.0 – 0.0] ^{A}	0.0 [0.0 – 0.0] ^{A}	0.632
Lung	0.0 [0.0 – 0.0] ^{A}	0.0 [0.0 – 14.2] ^{A}	0.0 [0.0 – 0.0] ^{A}	0.0 [0.0 – 0.0] ^{A}	0.0 [0.0 – 0.0] ^{A}	0.0 [0.0 – 0.0] ^{A}	0.0 [0.0 – 0.0] ^{A}	0.0 [0.0 – 0.0] ^{A}	0.690
Liver	0.5 [0.0 – 10.1] ^{A}	0.0 [0.0 – 0.2] ^{A}	0.7 [0.2 – 2.4] ^{A}	0.9 [0.1 – 2.7] ^{A}	0.1 [0.0 – 1.5] ^{A}	2.1 [0.7 – 12.4] ^{A}	0.1 [0.0 – 1.5] ^{A}	0.0 [0.0 – 0.0] ^{A}	0.076
Left arm	1.3 [0.0 – 18.5] ^{AB}	9.2 [0.1 – 19.9] ^{AB}	1096.0 [0.7 – 2060.2] ^{A}	112.3 [9.7 – 245.1] ^{A}	0.2 [0.0 – 2.1] ^{AB}	1.8 [0.5 – 3.9] ^{AB}	0.6 [0.0 – 2.5] ^{AB}	0.0 [0.0 – 0.0] ^{B}	0.006
Right arm	7.5 [1.6 – 20.9] ^{ABC}	0.7 [0.0 – 751.0] ^{ABC}	291.6 [70.2 – 583.3] ^{AB}	1401.1 [1171.1 – 1864.8] ^{A}	0.8 [0.0 – 4.6] ^{BC}	4.1 [0.0 – 10.7] ^{ABC}	0.1 [0.0 – 1.4] ^{BC}	0.0 [0.0 – 0.0] ^{C}	< 0.001
Left leg muscle	2.5 [0.0 – 69.5] ^{AB}	39.0 [0.1 – 204.5] ^{AB}	410.6 [24.0 – 547.5] ^{A}	1044.6 [531.0 – 1335.1] ^{A}	1.1 [0.6 – 2.1] ^{AB}	0.8 [0.0 – 3.4] ^{AB}	0.6 [0.0 – 1.1] ^{AB}	0.0 [0.0 – 0.0] ^{B}	0.001
Right leg muscle	2.9 [0.0 – 10.2] ^{AB}	1.3 [0.0 – 201.2] ^{AB}	783.4 [79.7 – 2502.9] ^{A}	449.8 [283.2 – 703.2] ^{A}	1.6 [0.2 – 3.1] ^{AB}	1.4 [0.0 – 2.9] ^{AB}	0.5 [0.1 – 1.4] ^{AB}	0.0 [0.0 – 0.0] ^{B}	0.002
Left back muscle	3.0 [0.0 – 4.8] ^{ABC}	65.5 [0.6 – 227.6] ^{ABC}	775.9 [95.2 – 2894.6] ^{A}	334.0 [224.0 – 723.2] ^{AB}	0.3 [0.0 – 1.3] ^{BC}	3.4 [0.1 – 10.1] ^{ABC}	1.7 [0.0 – 1.9] ^{ABC}	0.0 [0.0 – 0.0] ^{C}	< 0.001
Right back muscle	2.2 [0.0 – 7.7] ^{AB}	1.1 [0.0 – 204.3] ^{AB}	65.9 [32.9 – 227.7] ^{A}	333.5 [60.8 – 1444.0] ^{A}	0.9 [0.0 – 1.5] ^{AB}	2.4 [0.0 – 5.4] ^{AB}	1.2 [0.0 – 1.7] ^{AB}	0.0 [0.0 – 0.0] ^{B}	< 0.001

Medians followed by the same letter on the same line presented no difference between them by Tukey Test (P > 0,05). Kruskal-Wallis Test.

Table 3 Fecundity level (%); Medium number of neonates/female and Medium number of parasitary load by qPCR for *T. gondii* of neonats, by evaluated group. Botucatu-SP, Brazil, 2012

Group	Fecundity[1] (%)	qPCR Neonate[2] (parasite·mL^{-1})	Medium number of neonates/female
1	66.6^{a}	4.9 ± 4.4^{ab}	10.50
2	33.3^{b}	7.4 ± 10.6^{a}	9.00
3	83.3^{a}	1.4 ± 2.8^{c}	9.40
4	33.3^{b}	1.5 ± 1.7^{bc}	9.00
5	66.6^{a}	1.7 ± 2.4^{bc}	11.50
6	33.3^{b}	1.4 ± 1.2^{bc}	5.50
7	83.3^{a}	1.0 ± 1.4^{c}	10.20
8	83.3^{a}	0.0 ± 0.0^{c}	10.00
P	< 0.001	< 0.001	

[1] Percentages followed by the same letter on the same column were not different between them by χ^2 test (P > 0.05)

[2] Medium followed by the same letter on the same column were not different between them by Tukey test (P > 0.05).

Table 4 Median of cerebral cysts count, by examined group. Botucatu-SP, Brazil, 2012

Group	Cerebral cysts in female [2]
1	0.0 ± 0.0^{b}
2	10.0 ± 10.9^{b}
3	10.0 ± 16.7^{b}
4	26.7 ± 10.3^{a}
5	0.0 ± 0.0^{b}
6	0.0 ± 0.0^{b}
7	0.0 ± 0.0^{b}
8	0.0 ± 0.0^{b}
P	< 0.001

[1] Percentages followed by the same letter on the same column were not different between them by χ^2 test (P > 0.05)

[2] Medium followed by the same letter on the same column were not different between them by Tukey test (P > 0.05).

Table 5 Medium numbers of neutrophils, lymphocytes and macrophages (± standard deviation) per mm² of small intestine mucosae of vaccinated and challenged female rats (groups 1, 3, 5); non-vaccinated and challenged (groups 2, 4, 6); vaccinated (7) and control (8). Botucatu-SP, Brazil, 2012

	Groups				
	1	2	3	4	
Small intestine cells					
Neutrophils	126.5 ± 31.9^{AB}	77.2 ± 21.6^{AB}	104.9 ± 51.9^{AB}	67.9 ± 25.3^{B}	
Lymphocytes	737.6 ± 228.2^{AB}	415.1 ± 73.0^{C}	737.6 ± 162.4^{AB}	564.8 ± 126.0^{BC}	
Macrófages	18.5 ± 20.2^{A}	18.5 ± 16.5^{A}	12.3 ± 9.5^{A}	12.3 ± 9.5^{A}	
	Groups				P
	5	6	7	8	
Small intestine cells					
Neutrophils	142.0 ± 51.9^{A}	108.0 ± 39.6^{AB}	98.8 ± 27.9^{AB}	61.7 ± 30.2^{B}	0.005
Lymphocytes	597.1 ± 150.4^{BC}	496.9 ± 88.9^{BC}	904.3 ± 119.2^{A}	569.4 ± 115.1^{BC}	< 0.001
Macrófages	18.5 ± 11.7^{A}	18.5 ± 11.7A	24.6 ± 19.1^{A}	12.3 ± 9.5^{A}	0.775

Medium followed by the same letter on the same line presented no difference between them by Tukey Test (P > 0,05). Variance analysis.

T. gondii parasite load was lower in brain, heart and muscle from the vaccinated animals. However, vaccine had not blocked the infection course. The parasite loads were similar between neonates from vaccinated and non-vaccinated rats, but the irradiated vaccine with *T. gondii* tachizoites determined better efficacy results on birth rates and litters' size. In all groups samples of spleen and liver were negative. The higher positive results by PCR were found in muscle, followed by brain and heart tissues. The high parasite loads were found on groups challenged by oocysts, followed by the ones that were challenged by bradizoites.

In the groups challenged by tachizoites, the high parasite load was found in liver tissues. Parasite load of vaccinated and challenged female rats was low in brain tissues. *T. gondii* DNA was not found in brain tissues of rats that were only vaccinated.

Vaccine induced satisfactory results on parasite load of neonates and females. Brain cysts reduced counts were also verified.

Vaccinated animals presented better local and cellular immune response at small intestine of evaluated animals.

Conclusions

The results of the present study indicate that immunization with irradiated tachyzoites of *T. gondii* induced reduction of the parasitic load in most organs analyzed, although not prevent the establishment of infection with the parasite. And also, the immunization showed a favorable effect on the birth rate and litters' size.

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Effect of Animal Welfare on Reproductive Parameters in Dairy Cows

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Summary: The aim of this study was to assess the effect of animal welfare(AW) on reproductive parameters in dairy cows. The work was performed in 25 livestock units (UPAS) dairy cows of Tizayuca in Mexico. We evaluated the comfort of the cows (facilities management, health, nutrition and behavior). We used a questionnaire called Score for cow comfort on the Dairy Farm. Using breeding records fertility was assessed taking into account aspects such as calving interval, delivery, conception, services per conception, conception dose, percentage of waste for reproductive problems and age at first calving of primiparous cows. Subsequently, ordered data comfort and fertility data in the program JMP3. 1. 2 SAS Institute, with which a correlation was performed, comfort-fertility. With the data collected was performed fertility descriptive statistics and finally, analyzed the type of hormones that are used. The UPAS that took maximum comfort, had an average value of 17. 5 fertility and those with minimum of comfort, had a minimum level of comfort, was 8. 63, the significance level was(P = 0. 13). We obtained a R = 0. 09 indicating that fertility is explained in 09 1 in the comfort and. 91 for variables other than the comfort, also a positive relationship between the comfort found in animals and fertility. Of the 14 work UPAS valued only under conditions of cow comfort and the other 11 have minimum comfort conditions. Services per conception, conception dose, interval from calving to calving, age at first childbirth, were elevated with respect to the optimum, however, not reach values that cause problems, the percentage of waste for reproductive problems, indicated serious problems. The interval from calving to conception was all that was within the optimal values based on average of descriptive statistics. In conclusion, we need to AW, in UPAS analyzed, so it is recommended to raise awareness of staff working in the UPAS of the importance of AW, on the productivity of animals.

Introduction

The goal of every dairy farmer is to produce as much milk as possible under a minimal cost, but to achieve this goal it is necessary to provide the necessary environments, which should promote animal welfare. Currently, when considering animal behavior in the UPAS, production can be improved, since knowledge of BA can be applied to programs feeding, breeding, facility design, handling and transportation of animals (Etol, 2004, Ortega and Gómez, 2006).

Vellum et al(2004) observed that cows spend most of the time to behaviors that are classified as maintenance, prevailing time spent resting, ruminating and social behavior, mainly the social licking, are cows that are in a welfare state, a condition attributable among other factors, housing conditions and temperature. Animals that are crowded, they often develop stereotypical behaviors, severely affecting productivity (Vickery and Mason, 2005). The aim of this study was to assess the effect of animal welfare (AW) on reproductive parameters in dairy cows.

Material and methods

This work was performed in 25 UPAS Dairy cows of Tizayuca in México. Comfort was assessed visually in the

UPAS found in terms of facilities, management, health, food, cleanliness and behavior, this evaluation was subjective and was based on a questionnaire called the Score for cow comfort on the Dairy Farm, spent two hours a day for each UPA was revised cleaning the drinkers, feeders, beds, floors, was observed the treatment provided to animals by managers; noise that was in each UPA, we counted the number of beds of drinkers and feeders, it was verified the material of the beds and floors, was measured with a tape measure the width and length of feeders, beds and walkways, with environmental thermometer, we measured the temperature in each UPA, were verified the light periods and finally assessed the body condition. Points earned by UPA, were emptied into an Excel spreadsheet in order to assess that obtained UPAS comfort and what fell into the category of less comfort. Using breeding records fertility was assessed, taking into account birth interval from calving, calving to conception, services per conception, conception dose, percentage of waste for reproductive problems and age at first calving, primiparous cows only. Reproductive data were recorded in Excel spreadsheet. Subsequently, ordered data comfort and fertility data in the program JMP3. 1. 2 SAS Institute, with which a correlation was performed, comfort-fertility. With the fertility data collected, we conducted a descriptive statistics UPAS together. Reproductive parameters were compared for each of the UPAS with those reported in the literature. Finally, we evaluated the type of hormones used in each of the UPAS.

Results and discussion

The UPAS that took maximum comfort, had an average value of 17. 5 fertility and those with minimum of comfort, had a minimum level of comfort, was 8. 63, the significance level was ($P = 0. 13$). We obtained a $R^2 =$ 0. 09 indicating that fertility is explained in 09 1 in the comfort and. 91 for variables other than the comfort, also a positive relationship between the comfort found in animals and fertility. Of the 14 work UPAS valued only under conditions of cow comfort and the other 11 have minimum comfort conditions. Services per conception, conception dose, interval from calving to calving, age at first childbirth, were elevated with respect to the optimum, however, not reach values that cause problems, the percentage of waste for reproductive problems, indicated serious problems. The interval from calving to conception was all that was within the optimal values based on average of descriptive statistics.

It has been reported in several studies (Xolalpa et al. , 2003, Cordova-Izquierdo et al. , 2010) that the existence of BA in UPAS should be taken into account various environmental and management factors, the results obtained in this work, prove it. Ortega and Gomez (2006) indicated that state that the proper handling of animals, especially from an early age may prevent them from developing their fear of humans, so it is very important to train those responsible for their management not to carry out aggressive practices, which often are unnecessary in routine work in the UPA and also affect the AW of animals.

In conclusion, we need to AW, in UPAS analyzed, so it is recommended to raise awareness of staff working in the UPAS of the importance of AW, on the productivity of animals.

Conclusions

We need to AW, in UPAS analyzed, so it is recommended to raise awareness of staff working in the UPAS of the importance of AW, on the productivity of animals.

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Detection of *vvhA* Gene of *Vibrio vulnificus* and *trh* Virulence Genes of *Vibrio parahaemolyticus* from Samples of *Ostrea edulis* Collected from the Black Sea using a PCR-Based Method

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Summary: *Ostrea edulis*, also known as the European flat oyster, is a well-known freshly-consumed food product, pertaining to the human diet for centuries. *Vibrio parahaemolyticus* and *Vibrio vulnificus* represent two foodborne pathogens with pronounced lethality, usually found to be optimally developing in estuaries, where this oyster species is normally collected for food purposes, in the end the risk for the consumer's health being a high one through the consumption of this food product.

In this study, a number of 200 samples of European flat oyster was collected and analyzed using a polymerase chain reaction (PCR)-based method, in order to detect the presence and isolate the targeted foodborne pathogens. In a secondary step, the PCR-based method was used to reveal the presence of the specific virulence genes, *vvhA* for *V. vulnificus*, and *trh*1 and *trh*2 genes of *V. parahaemolyticus*.

The results revealed that 46 samples were positive for the presence of *V. parahaemolyticus*, of which 23 were positive for the presence of *trh*1 and 18 for *trh*2. Considering *V. vulnificus*, 38 samples out of 100 were positive, of which 34 presented the *vvhA* virulence gene. These data suggest that the European flat oysters represent a high risk for the consumer's health, not only through the presence of two of the most important foodborne pathogens in Europe and worldwide, but mainly through the presence of the specific virulence genes.

Introduction

Vibrio spp. are usually present in the sea water, determining infection in humans and producing illness in different aquatic species, further on contaminating the food products. Through the morbid consequences, *Vibrio* spp. determines a series of problems considering the mollusk exploitation units. *V. parahemolyticus* and *V. vulnificus* are frequently involved in determining human gastroenteritis, further on these developing serious illnesses, much worse than those determined by viruses [1, 5, 10, 13].

Vibrio parahaemolyticus is a Gram-negative, non-spore-forming rod-shaped bacterium, with a high motility in liquid media, due to its polar filament. It determines one of the most severe forms of gastroenteritis related to aquatic food products, being largely distributed in areas such as estuaries and revealing a higher prevalence during the warm season [15, 27]. This foodborne pathogen is frequently isolated from oysters, due to their specific feeding mechanism, by water filtration, a process in which the bacterial concentration becomes much higher than that of the water they live in [4, 24, 25].

The illness is usually related to the consumption of fresh oysters and other aquatic food products [4, 18]. The epidemiologic research performed until now showed a direct relation between the thermostable hemolysin (TDH) and a variant of the TDH, called TRH, both associated with the pathogenicity of *Vibrio parahaemolyticus* [16]. Both hemolysins are regarded as virulence factors specific to this species [10]. The TRH was studied along with the specific virulence genes, *trh*1 and *trh*2, the research revealing that through hybridization, the weak signal of the test after *trh* identification was due to a variant of this gene, further on called *trh*2, while *trh* became *trh*1. This variant, along with *trh*1 determined Kishishita et al. (1992) [14] to conclude that only the strains which possess both genes could be considered to reveal virulence potential on the infected hosts.

Vibrio vulnificus is transmitted through the contaminated food consumption, being responsible for a large number of deaths caused by aquatic food products [6, 11, 19]. This bacterial species was isolated in the water as well as in the sediments and from a large variety of aquatic products and fish [7, 9, 20]. It elaborates an extracellular hemolysin, genetically regulated through the presence of *vvhA*, which contribute to iron's release through the hemolytic activity, thus being partially responsible for the cytotoxic activity of this bacterial species [25]. The direct examination of hemolysin's effects on host cells resulted in the presence of a toxin which determined the increase of vascular permeability, the endothelial cells' apoptosis, and the induction of nitric oxide synthase, while an increased production of nitric oxide was observed along with a potential elimination of neutrophils from the bloodstream [8, 10, 12]. The foodborne illnesses due to these two species present a seasonal pattern, with a high number of cases during the summer. The prevalence of these species in food products is usually low [2, 24].

The aim of this paper was to reveal the presence of *vvhA* pertaining to *V. vulnificus* and *trh*1 and *trh*2, as a

secondary step to the isolation of *V. parahaemolyticus*, from European flat oysters (*Ostrea edulis*) samples, collected from the Black Sea.

Material and methods

A total number of 200 samples consisting of European flat oysters were directly collected from the unit and aseptically packed. These were afterwards safely transported to the laboratory, being maintained continuously at 3 – 5℃. They were subjected to analysis in 3 h after the collection moment.

The oysters were prepared and the content was extracted and homogenized, afterwards being obtained 1∶1 and 1∶10 dilutions in alkaline buffered water (ABW), through their introduction in 50 and 180 ml ABW, respectively. The samples (0.2 g from 1∶1 dilution and 0.1 ml from the decimal dilution) were distributed in plates with agar containing 1% triptone and 3% sodium chloride, in duplicates. These plates were further on incubated at 37℃ for 18 – 24 h [23].

DNA extraction. The bacterial cultures and the oyster samples were centrifuged at 5000 rpm for 10 min and the sediment was collected using a QIAamp DNA kit. The DNA quantity and its purity were determined using spectrophotometry, obtaining the percentages for 260/280 nm. The concentrated DNA (a result of the ethanol precipitation) was subjected to vacuum evaporation, through centrifugation. The sediment was suspended again in 50 or 100 μl of Tris-EDTA buffer solution (pH = 8; 10 mM Tris, 1 mM EDTA) and incubated at 65℃ for 10 min in order to solubilize the DNA. The concentrated DNA was stored at –20℃.

PCR for *V. vulnificus*. The oligonucleotides were derived from the cytolysin structural gene, *vvhA*. This is located in a region previously used to collect specific sequences. The Primers Express software was used to select the TaqMan sequence (5'-CCG TTA ACC GAA CCA CCC GCA A-3'), the first (5'-TGT TTA TGG TGA GAA CGG TGA CA-3') as well as the last (5'-TTC TTT ATC TAG GCC CCA AAC TTG-3') for the specific sets of primers.

The polymerase chain reaction included the use of reagents and the specific TaqMan technique. This test is based on the fluorescence emitted by the cleavage of a dyeing substance during the PCR. Its fluorescence is discarded in the intact sequence due to the proximity of the natural dyeing substances. The amplification mixes (50 μl) contained different DNA concentrations (3.0 μl), TaqMan A buffer solution (5 mM $MgCl_2$; 200 μM dATP, dGTP, dCTP, and 400 μM dUTP), a fluorogenic TaqMan sequence (0.25 μM), primers (0.90 μM of each) and the DNA AmpliTaq Gold polymerase (2.5 U). The reactions were performed using a Roche 2.0 system, while the data were analyzed using the sequential detection software GeneAmp 5700.

The product obtained through the PCR was detected through monitoring the increase of the fluorescent signal, generate by the 6-carboxyfluorescein labeled sequence [3,22].

Detection of *V. parahaemolyticus* virulence genes. In order to detect *trh* genes, a PCR test was applied using R2 (5'-GGCTCAAAATGGTTAAGCG-3') and R6 primers (5'-CATTTCCGCTCTCATATGC-3'). The positive samples were further on examined for the presence of either *trh*1 or *trh*2, through DNA hybridization. The specific sequences for *trh*1 and *trh*2 are represented by DNA fragment of 334 bp and 419 bp, isolated from recombinant plasmids, and labeled with dCTP 32P. The hybridization was realized through nitrocelulozic membranes, in a 50% formamide solution. The signals of the hybridization test were visually interpreted. The strong and further on weak signals were interpreted as *trh*1 positive while the weak and further on strong signals were interpreted as positive for *trh*2 [26].

Results and discussions

A number of 46 samples were positive for *V. parahaemolyticus* while 38 samples were positive for *V. vulnificus*. From the 46 *V. parahaemolyticus*-positive samples, 23 were *trh*1-positive and 18 were *trh*2-positive. From the 38 *V. vulnificus*-positive samples, 34 revealed the presence of *vvhA* (Table 1).

Table 1 Results of *V. parahaemolyticus* and *V. vulnificus* analysis and their specific virulence genes

Species	Total number of analyzed samples	Number of positive samples	Number of *trh*1 positive samples	Number of *trh*2 positive samples	Number of *vvhA* positive samples
V. parahaemolyticus	100	46	23	18	–
V. vulnificus	100	38	–	–	34

This step aimed to determine the virulence potential of *V. parahaemolyticus* and *V. vulnificus*. These were isolated from European flat oyster samples, originating in the Black Sea. The presence of *trh*1 and *trh*2 genes, as well as *vvhA* gene, was regarded as a measure of the virulence potential for the isolated strains. These data

demonstrate the existence of a high risk for the consumers' health, due to the presence of the two foodborne pathogens, as well as their specific virulence genes.

Miwa et al. (2005) [16] had 100% positive results for *V. parahaemolyticus* presence in the analyzed mollusks samples, while Yamazaki et al. (2010) [26] showed that 45 of their analyzed samples were positive for *trh*1 and 50 for *trh*2. Nordstrom et al. (2007) [17] also showed that from a number of 117 samples, 74 were positive for both genes.

Considering *V. vulnificus*, the scientific data showed a high number of samples that can represent a high risk of contamination for the consumers, the same result being discovered by O' Neill et al. (1992) [21]. They demonstrated the presence of *V. vulnificus* in 25 out of the 66 analyzed samples. Also, recently, in 2010, Chen et al. [3] demonstrated that *V. vulnificus*, along with *V. parahemolyticus*, were present in 67 out of 109 and 122 analyzed samples, respectively.

Conclusions

European flat oysters obtained in specialized exploitation units from the Black Sea shore areas can present a potential risk for consumers' health, due to the proven presence of the two lethal foodborne pathogens. Through the feeding system based on water filtration process, oysters can reach higher concentration of the two foodborne pathogens, in comparison to the water they live in.

The virulence genes' presence shows a high risk for the consumers, since oysters are traditionally consumed fresh. Nevertheless, studies concerning their detection can be usefully completed with others concerning the quantitative presence of these species, the latter being necessary for the confirmation of the risk's existence.

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Impact of Selected Climate Parameters on the Foot Pad Health Status of Turkey Poults

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Summary: It is commonly known, that the climate of the environment of productive livestock influences its well-being and productivity. Therefore the Order on the Protection of Animals and the Keeping of Production Animals (German designation: Tierschutz-Nutztierhaltungsverordnung, TierSchNutztV) claims in the General Requirements for Animal Husbandry Systems (§3(3) 2.), that "barns must be sufficiently thermally insulated and equipped in such a way, that circulation, dust level, temperature, humidity and gaseous concentrations in the air must be kept in a range that is unharmful for animals" (translated from German). In a previous field study carried out from 2007 to 2009, 11.860 turkeys were examined, amongst other parameters, concerning foot pad alterations. It then became evident that epithelial necrosis was diagnosed in 45% of the fattening turkeys at the early age of six weeks. Multiple factors have been linked to cause foot pad dermatitis. Aim of the present field study was to investigate possible connections between climate parameters and the occurrence of foot pad alterations during the early rearing phase. Altogether 2.681 beak-trimmed British United Turkeys 6 poults from twelve commercial farms were examined twice to that effect during generally two rearing periods. In addition to continuously recorded air temperature and humidity, ammonia and dust concentration were registered on each day of the examination. Dependencies on the foot pad health status could be detected concerning starting temperature ($P < 0.001$), temperature measured one week before the second examination ($P = 0.004$), humidity and gaseous ammonia concentration ($P < 0.001$). The results show that climate parameters, which can also be easily assessed by the farm manager, can give valid information about frequency and severity of foot pad alterations in turkey poults during the early rearing phase.

Introduction

Foot pad dermatitis [FDP] is commonly found in turkey flocks. This medical condition is described by an inflammatory to necrotic state of the plantar skin of the metatarsal and/or digital foot pads. As cause for the occurrence many factors are associated, e. g. poor litter condition and therefore a high litter moisture (Spindler, 2007; Abd El-Wahab et al., 2011; Wu and Hocking, 2011), exposition durance (Berk, 2007; Krautwald-Junghanns et al., 2011; Schumacher et al., 2012), and stocking density (Hafez et al., 2005). Foot pad alterations appear as well in turkeys and broilers and because especially in severe cases, they may cause discomfort and pain for the birds (Mayne et al., 2006; Berk, 2007) they require arrangements for prevention and attenuation (Kamphues et al., 2011). A successful early rearing phase sets the basis for the following fattening phase. To achieve the mentioned, one of the premises is an ideal designed climate (Berk, 2002). The Order on the Protection of Animals and the Keeping of Production Animals (German designation: Tierschutz-Nutztierhaltungsverordnung, TierSchNutztV) claims in the General requirements for Animal Husbandry Systems [§3(3) 2.], that "barns must be sufficiently thermally insulated and equipped in such a way, that circulation, dust level, temperature, humidity and gaseous concentrations in the air must be kept in a range that is unharmful for animals" (translated from German). Aim of the present field study was to analyse the foot pad condition during the early rearing phase up to five weeks of life and to scan possible connections with climate parameters as gaseous ammonia content, dust level, air temperature and humidity.

Material and methods

From July 2010 until January 2012, altogether twelve German commercial turkey farms with beak trimmed turkeys solely of the strain British United Turkeys [B. U. T.] 6, were visited twice during the early rearing period [days 3 – 5 (shortly after delivery from the hatchery) and days 22 – 35 (shortly before relocation)]. This scheme was in general repeated in a second rearing period. Air temperature and humidity as well in the stables as on the outer environment, were recorded continuously on an hourly basis with thermologgers (LogBox RHT, B + B Thermo-Technik GmbH, Germany). Gaseous ammonia in ppm (Pac III E/S,

DrägerSensor XS EC NH3 Dräger Safety AG & Co. KGaA, Germany) and dust level examinations in mg/m^3 (DustTrakTM Aerosol Monitor, Model 8520, TSI Incorporated, USA) took place during the visits. In general 60 randomly picked turkey poults (n = 2.681 [1481 male and 1.200 female]) were examined per visit concerning especially the foot pad condition. Due to the field conditions it became necessary to modify the scoring system for the foot pad condition established by Mayne (2005) and Hocking et al. (2008) according to Krautwald-Junghanns et al. (2009). Altogether five categories were used to describe foot pad alterations: 0 = no abnormality detected: surface of the skin of the foot pads shows no alterations; 1 = hyperkeratosis: moderate hypertrophy of the plantar skin; reticulate scales are elongated, but not dark coloured; 2 = high-grade hyperkeratosis with crusts of dirt: pronounced hypertrophy of the plantar skin; adhesive dirt cannot be removed without damaging the plantar skin; after manipulation bleeding tendency; 3 = superficial lesions, epithelial necrosis: dark coloured necrosis of (elongated) reticulate scales; 4 = profound lesions of the plantar skin, foot abscess, or both: ablation of the outer layer of the epidermis.

In addition wide-ranging collections of data concerning livestock husbandry and management were carried out.

Statistical Analysis

The statistical analysis took place in collaboration with the Statistical Consulting Unit, Department of Statistics, Ludwig-Maximilians-University, Munich, Germany under the direction of Prof. H. K chenhoff. For the statistical evaluation of the data the programming language and environment for data analysis and graphics R, version 2.15.1, was used (R Core Team, 2012). Results were considered significant if the P value was lower than 0.05. For the survey on independency the univariate Fisher-test was used. Because performed statistical correlations (Kendall: r = 0.798 and Spearman: r = 0.835) concerning the comparison between the right and the left foot of each examined turkey poult showed the same marginal distribution for both feet, the calculations were carried out with the data from the right side.

Results and discussion

At the early age of 3 – 5 days first alterations of the foot pads were diagnosed. In 76.9% (±SE 7.03) of the examined turkey poults no skin alterations were detected. 19.9% (± SE 5.99) were diagnosed with a mild hyperkeratosis of the category 1 during the days 3 – 5. High grade hyperkeratosis in the means of category 2 was found in 3.2% (±SE 1.63). At the age of 22 – 35 days 70.8% of the examined poults showed alterations of the foot pads such as hyperkeratosis (15.2% ± SE 3.49), hyperkeratosis with adhesive dirt (33.0% ± SE 6.36) and epithelial necrosis (22.6% ± SE 6.86). Deep lesions were diagnosed in 0.2% of the examined birds.

A great heterogeneity, especially during the days 22 – 35, occurred between the different examined farms concerning gaseous ammonia content (0.0 – 63.4 ppm). A relationship could be detected between the gaseous ammonia content and the foot pad health status ($P < 0.001$). Therefore the foot pad health was worse when the average ammonia concentration exceeded the average (for day 3 – 5: 0.4 ppm and days 22 – 35: 12.4 ppm). The gaseous ammonia concentration significantly depends on litter moisture and the pH-value of the litter (Reece et al., 1979; Elliot and Collins, 1982) and therefore is a good indicator for an ideal or suboptimal litter and ventilation management. Also the dust levels (0.03 – 1.17 mg/m^3) varied between the twelve farms and the days of examination but no difference could be detected concerning the poults' age, different areas of measurements or rearing period. Humidity averaged between 42.1% and 71.9%, air temperature averaged 26.6℃. As well as for the starting-temperature ($P < 0.001$) as for the seven-day temperature ($P < 0.05$) a connection between the foot pad health status, concerning temperature could be assessed. The results show that the higher the temperature was and the lower the humidity the better the results of the examined foot pads. In reverse this most probably leads to a better, respective dryer litter quality which is seen as the factor with the highest impact on the occurrence of foot pad dermatitis.

For more detailed results on foot pad dermatitis in poults during the early rearing phase see:

Bergmann, S., Ziegler, N., Bartels, T., Hübel, J., Schumacher, C., Rauch, E., Brandl, S., Bender, A., Casalicchio, G., Krautwald-Junghanns, M.-E., Erhard, M. H. (2013). Prevalence and severity of foot pad alterations in German turkey poults during the early rearing phase. Poult. Sci (accepted)

Ziegler, N., Bergmann, S., Hübel, J., Bartels, T., Schumacher, C., Bender, A., Casalicchio, G., Küchenhoff, H., Krautwald-Junghanns, M.-E., Erhard, M. H. (2013): Auswirkungen des Stallklimas auf die Fuβballengesundheit von Mastputen der Herkunft B. U. T. 6 in der Aufzuchtphase, Berl. Muench. Tieraerztl. Wschr. 2013 (accepted)

Schumacher, C., Krautwald-Junghanns, M.-E., Hübel, J., Bergmann, S., Mädl, N., Erhard, M. H., Berk, J., Pees, M., Truyen, U., Bartels, T. (2012): Effekte der Substratfeuchte im Futter- und Tränkebereich auf die Fuβballengesundheit von Mastputen der Herkunft B. U. T. Big 6 in der Aufzuchtphase, Berl. Muench.

Tieraerztl. Wschr. 2012; 125: 379-385

Conclusions

Especially during the first few weeks of life climate parameters can affect a successful upbringing and pioneer the condition for turkey poults for the following fattening phase. An adjusted climate management can have a positive impact on the foot pad health status because also other factors, e. g. litter condition, are most probable positively influenced.

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Analysis of Environmental Sources for *Staphylococci* in Milk of Dairy Cows

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Summary: The aim of this study was to analyze the potential infectious sources in the environment of lactating cows. For this purpose, we characterized the diversity of *Staphylococcus* species in a German dairy farm. Species present in the milk were compared with species from different locations in the direct environment of the cow. The examined herd consisted of 100 Holstein Friesian cows in a typical three-row cubicle house with rubber mats and slatted floor. Milk samples of each quarter of ten cows with somatic cell counts higher and lower than 100,000 cells/ml were screened. Sampling of cows and environment rotated weekly over a period of two months. In total, 120 milk samples and 158 environmental samples were analyzed bacteriologically by routine bacteriological diagnostics including biochemical differentiation. Additionally, we screened for Methicillin-resistance of *Staphylococcus aureus*, and tested this species for antimicrobial resistance against Cefquinom and Lincomycin. Data analysis and statistics were performed using the statistical software SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

Introduction

Mastitis is a common problem in dairy farming worldwide regarding animal health and economic aspects. Several causative pathogens are known, and especially *Staphylococcus* (*S.*) *aureus* and coagulase-negative *Staphylococci* are increasing in incidence (Pyörälä and Taponen, 2009). Coagulase-negative *Staphylococci* (CNS) have become the predominant pathogens causing bovine mastitis in many countries (Rajala-Schulzet al., 2004). Although CNS usually cause only subclinical or mild clinical mastitis (Taponen et al., 2006), they are harmful since they increase somatic cell count (SCC) in milk and may slightly decrease milk production (De Vliegher et al., 2005). Many known control measures such as milking hygiene, or culling of infected cows are not always efficient in preventing new *S. aureus* infections (Sommerhauser et al., 2003), indicating the complexity of the problem and the possibility of other sources of infection. Among all *Staphylococcus* species (spp.), including *S. aureus*, some strains seem to be more virulent than others (Taponen et al., 2009). To minimize the potential risk of infection, the knowledge of possible sources in the environment of cows and infection routes for *Staphylococci* is necessary.

Material and methods

Animals and practice on farm

The examined German Holstein herd consisted of 100 cows in a typical three-row cubicle house with rubber mats and slatted floor. The mats were cleaned two times a day, and limed every three days. During milking, the milker wear gloves and the teat ends were dipped with an iodine solution after milking. Milking personal disinfected the milking machine clusters after milking cows with clinical symptoms or known high SCC with more than 100,000 cells/ml. Milk samples of each quarter of ten multiparous cows (lactation 2 – 6) with known SCC higher and lower than 100,000 cells/ml were screened. Sampling of cows and environmental locations rotated weekly over a period of two months. In total, 120 milk samples and 158 environmental samples were available for evaluation.

Regarding environmental samples, 31 locations including areas with and without udder contact were sampled with a swab. Air samples were taken in the milking, the lying and the standing area using the MAS-100 Eco Air Sampler (Merck KGaA, Darmstadt, Germany). Furthermore, the used feed, the water and other possible mechanical and biological vectors were analyzed bacteriologically.

In addition to the milk samples, clinical symptoms of the cow's udder were recorded including edema signs and flakes or clods in the milk for every quarter. All samples were taken during milking and normal working processes.

Laboratory methods and statistics

All samples were taken in the evening and stored cool and dry at 4℃. After 12 hours, the microbiological routine analysis started including cultivation on a selective agar for *Staphylococci* (Baird Parker) and biochemical identification (API ID 32 STAPH, bioMérieux, France).

Additionally, we screened for Methicillin-resistance of *S. aureus*via Brilliance MRSA Agarand tested for antimicrobial resistance against Cefquinom and

Lincomycin the most often used antibiotics at the farm of the isolated *S. aureus* (n = 25) from milk and environmental samples. Data analysis and statistics were performed using the statistical software SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

With respect to the milk samples, 0.6 bacterial strains per milk sample were determined in average. In 53% of the samples, no *Staphylococci*, in 36% of cases one, and in 11% two or three *Staphylococcus* species were identified. In total, 73 *Staphylococci* were isolated belonging to more than eleven different *Staphylococci* species. For detailed evaluation, three classes of SCC were created: class I with SCC ≤ 100.000 cells/ml, class II with 100.001 < SCC ≤ 400.000 cells/ml and class III with SCC > 400.000 cells/ml. These three classes correlated with the occurrence of clinical signs of mastitis. Differences between the cell count classes considering the isolated species are shown in Fig. 1.

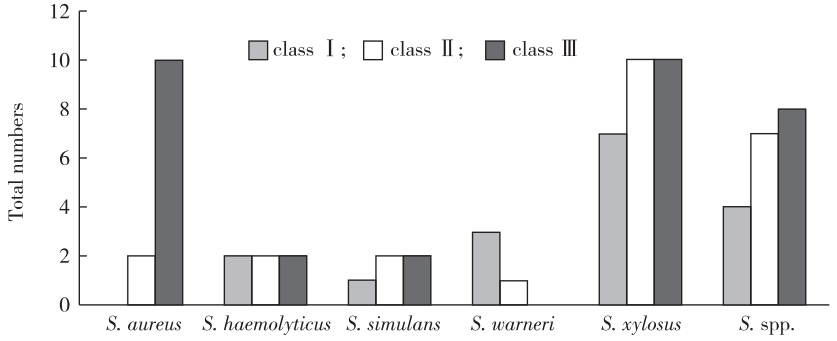

Fig. 1 Occurrence of *Staphylococci* species within milk samples (n = 120) classified by somatic cell count (SCC): class I with SCC ≤ 100.000 cells/ml (n = 36), class II with 100.001 < SCC ≤ 400.000 (n = 36), and class III with SCC > 400.000 cells/ml (n = 48).

As predominant species, *S. xylosus* (37%) and *S. aureus* (17%) were most often isolated in milk samples. Whereas *S. aureus* was found in twelve quarters with ten of them showing clinical signs, *S. xylosus* was isolated in 23 healthy quarters and in four affected quarters only. From the milk samples, 17% and 25% of isolated *S. aureus* were resistant against Lyncomycinor Cefquinom, respectively. All *S. aureus* isolates were sensitive for Methicillin.

Referring to the environmental samples, 0.9 bacterial strains were determined in average. In 49% of the samples, no *Staphylococci*, in 27% one and in 18% two *Staphylocci* were isolated. The highest contamination with five different *Staphylococci* was observed in samples by the air sampler, followed by four different *Staphylococci* at the slatted floor. In total, 136 *Staphylococci* were isolated belonging to 15 different species. Most frequently present was *S. chromogenes* (29%), followed by *S. xylosus* (12%), *S. aureus* (10%) and *S. haemolyticus* (7%). *Staphylococcus chromogenes* was isolated from many origins, but most often identified on the mats. *Staphylococcus aureus* was detected on the rubber mats, on the slatted floor, and in the air samples in the milking area. *Staphylococcus xylosus* was mainly isolated in dust reservoirs, on rubber mats and in the air samples in the milking area. From the environmental samples, 15% and 23% of the isolated *S. aureus* were resistant against Lyncomycinor Cefquinom, respectively. All *S. aureus* isolates were sensitive for Methicillin. The Cefquinom-resistant *S. aureus* were found three times on mats, and three times in air samples, and the Lyncomycin-resistant *S. aureus* were detected two times on mats and on floor.

The bacteriological results from the milk samples were compared to environmental samples under special emphasis on locations with or without direct udder contact (Table 1).

All *Staphylococci* were most frequently found in the environment with no contact to the udder. Although *S. chromogenes* was found in the environment with direct udder contact, this species was not isolated from milk samples. In contrast, *S. simulans* was isolated from milk samples, but not from environmental samples from locations with direct udder contact.

Table 1 Species distribution for *Staphylococcaceae* in milk samples (n = 120) andenvironmental samples (n = 158) in total and in% (in parentheses)

	Staphylococci species from milk samples in total and in%	*Staphylococci* species from environmental samples in total and in%	
		with udder contact	no udder contact
Staphylococcaceae	73	135	
S. aureus	12(16.0%)	5(3.7%)	8(5.9%)
S. chromogenes	0(0)	16(11.9%)	23(17.0%)
S. xylosus	27(37.0%)	3(2.2%)	13(9.6%)
S. hämolyticus	6(8.0%)	2(1.5%)	8(6.0%)
S. simulans	5(7.0%)	0(0)	2(1.5%)
S. warneri	4(6.0%)	1(0.7%)	5(4.0%)
S. spp.	19(26.0%)	11(8.0%)	38(28.0%)

Discussion

In this study, *S. simulans* and *S. xylosus* were discovered as the most present CNS in udder quarters affected by mastitis. While *S. xylosus* was found mostly in non-udder contact areas, *S. simulans* was rarely found in the environment, indicating that other reservoirs were more important as source of infection. In studies from Taponen et al. (2006) and De Vliegher et al. (2011), *S. simulans* and *S. chromogenes* were the most frequently isolated CNS from clinical and subclinical bovine mastitis. However, in our study *S. chromogenes* was not found in milk samples, although it was present in the environment. It is described in literature, that *S. chromogenes* dominated in first lactation, while *S. simulans* was more often isolated in subsequent lactations (Rajala-Schulz et al., 2004; De Vliegheret al., 2003; Jarp, 1991). The lack of *S. chromogenes* can therefore be explained by the higher lactation numbers of cows sampled in this study.

Staphylococcus aureus was often found in the environment on locations with or without direct udder contact. Multiple reservoirs of *S. aureus* were described in other studies, for instance Smith et al. (2005) detected *S. aureus*from milking machine clusters, farm personnel and from a cubicle partition. Capurro et al. (2009) indicated hock skin as an important reservoir for *S. aureus*. In our study, the environment was identified as reservoir for *S. aureus*, *S. xylosus* and *S. haemolyticus*, which might be related to the occurrence of mastitis.

Conclusions

In this study, *S. simulans* and *S. xylosus* as most present CNS, and *S. aureus* were identified as possible agent scausing mastitis. However, even in healthy quarters, different species were isolated, indicating also healthy cows as potential reservoirs. Whereas most CNS were found mainly in environment samples from locations without direct contact to the udder, *S. aureus* was frequently in all environmental samples both with and without udder contact. A strict hygiene and disinfection regime is therefore necessary in the whole stable environment, including also areas without direct animal or udder contact.

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Effect of Climatic Factors on Reproduction of Cattle in the Mexican Tropics

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Summary: Reproductive behavior of cattle in the tropics, is achieved when the animals show their full reproductive potential, however, the conditions existing in agroecosystems may have limitations on the ultimate manifestation of that potential. The prolonged postpartum anestrus is a main factor limiting reproductive efficiency in cattle, particularly in Bos indicus, Bos taurus and Bos taurus/Bos indicus in tropical regions. Discusses various climatic factors that may determine the reproductive behavior of cattle in the tropics, such as temperature, wind, humidity, precipitation, solar radiation, light, clouds and atmospheric pressure.

Introduction

The reproductive performance of cattle in tropical conditions, depends largely on its adaptation to ambient weather conditions. There are animal health practices and structures that can alleviate the harmful effect of climatic factors of the tropical environment. Therefore, it is necessary to identify the limiting climatic factors and estimate their effect on reproductive performance. Climate is the most important environmental factor, when trying to raise animals in tropical environments (Montiel and Ahuja, 2005; Henshall, 2004; Hafez, 2000, Martin and Garcia, 1985).

Temperature:

It is the most important element that limits the type of animal that can be raised in a given region.

Heat Stress:

For all mammals is possible to define a zone of thermal comfort. The constancy of the heat losses due to peripheral vasodilatation, without that other mechanisms are in place. Beyond this zone, the evaporation of body fluids allows regulating the heat loss as: the outside temperature increases.

Wind:

It has been noted that in cattle Bos indicus the heat dissipation mechanism is the most important sweating, suggesting that the strong activity of the sweat glands of cattle Bos inducus and their short coat are responsible for this phenomenon.

Humidity:

The relative humidity, it may work in combination with rainfall or individually affect the expression of estrus (Villagomez et al., 2000).

Rush:

In cattle decreases the duration of estrus, in times where there is more rainfall and relative humidity, these conditions are present in the summer and autumn (Villagomez et al., 2000).

Solar radiation:

Solar radiation is closely related to the atmospheric temperature and the degree of cloudiness and thus, with precipitation (Shell et al., 1995).

Light:

The photoperiod mechanism controls the sexual cycle in some pets. However, it has a noticeable effect on reproductive performance of cattle (Hafez, 1972). But it

has been shown that there is a stronger association between photoperiod, temperature and heat stroke are the estrus(Villagomez et al. , 2000).

Cloudiness:

The extent and persistence of cloudiness exerts an indirect effect on the environment of the animal in warm climates. It can be used to calculate the levels of solar radiation and humidity. Therefore, indirectly indicates the periods of lack of comfort of the animals.

Atmospheric pressure:

The change in pressure that occurs between the different heights directly affects animals. Because of the pressure decrease, the animals show difficulties in meeting their oxygen requirements. In this situation, should increase the rate of hemoglobin. Furthermore, adaptation of the organism to the decrease in oxygen is performed also by increasing cardiac and respiratory frequency(Hafez, 2000 and Brosh et al. , 1998).

Results and discussion

Based on the authors' experience as a conclusion, are some points to consider to mitigate the effects of climatic factors on the reproductive behavior of cattle in the tropics.

Conclusions

· Provide a ventilation system that controls body temperature. Implement water sprinklers.

· Protection of animals against solar radiation, direct and indirect, through shadows or appropriate ceilings.

· Provide shade in feeding and drinking, to increase feed intake in animals under stress caloric.

· Provide water sprays.

· Provide animals, spray baths, in the hottest part of the day. Trying to keep animals with white fur, as they are the most easily absorb heat and therefore are less sensitive to heat stress.

· Develop genetically adapted animals, because they may be less sensitive to heat stress.

· Implementation of frozen embryos.

· Inseminate semen frozen in time less hot.

· Provide free areas in the Production Unit and shaded. Provide the required area per animal for comfort.

· Bathing females, pre-service and 3 – 5 days.

· Inseminate or service in less hot periods.

· Implement estrus synchronization programs, or to schedule service inseminations.

· Do not isolate females long before artificial insemination or service.

· Properly balance diets, providing the energy needed to offset declining intake presented.

· Reduce intake of fiber and protein and increase energy.

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Effect on the Boar Sperm Motility PRRS Virus

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Summary: The objective of the study was to evaluate the sperm viability of semen infected with PRRS viral particles and the effect of the Virus on the mobility of boar spermatozoa. The work was carried out in the laboratory of genetics and reproduction FMVZ-BUAP. Semen was obtained from the center of Insemination in Tecamachalco, Puebla. 5 Stallions were used. Each sample containing 1×10^6 spermatozoa, PRRS virus strain was ATCC-VR-2332(0, 102, 104 and 106 prints RNA/mL in triplicate), observed daily in the home; Hamilton Thorne®. Cells with Total Motility(MT)($P<0.05$) on days 1, 3, 5, 7 and 10 of the evaluation. Curvilinear Velocity(VCL) on day 1 and 7 to $10 \times 10 \times 6$ and 2 PI($P<0.05$) vs control. Velocity Straight Line(VSL) and Velocity Average Path(VAP) in day 1 day Postinfection(DPI) $10 \times^2$, $10 \times^4$ and $10 \times^6$;($P<0.05$) vs. the control. The Amplitude of the Lateral Head(ALH) on day 5 and 7 to 10×4 and 10×6 DPI($P<0.05$) vs control. The Progressive Motility(MP) on day 1 and 3 DPI to $10 \times 10 \times 4$ and 2($P<0.05$) vs control. In the matrix of correlations, observed a correlation($P \geq 0.001$) between VSL with VAP; VSL with VCL. Also between VCL and ALH. The VAP with ALH. The VSL with HLA; the STR with ALH and VCL str. Finally, found a relationship between the different types of sperm mobility arising out of the House, resulting in that the addition of PRRS Virus in boar semen affects the motility of sperm.

Introduction

Yaeger et al., (1993) reported that semen is a potential resource of PRRS virus transmission, between 1991 and 1992 observed between 200 – 250 pregnant females began to have a high incidence of abortions and stillbirths and pigs with respiratory symptoms, these authors detected the presence of PRRS virus in lung by lung-conjugated antibodies in fresh and artificial insemination found that had been the cause of the epidemic. They analyzed the volume of the ejaculate and color as well as the concentration, motility and sperm morphology, observed a decrease in volume while the concentration, color, mobility and morphology were within normal limits. Associated with the above Swenson et al. (1994) semen samples collected on days 8, 7 and 6 before infection with the virus and then infected intranasally a stud(106.5 units/ml) 7, 8, 14 and 21 days PI and analyzed the mobility, concentration, sperm morphology and found that motility and morphology parameters were within normal limits, likewise, detected the presence of PRRS virus in pigs from 7 dpi by antibodies conjugates after insemination with infected semen or not, found no difference in the number of piglets born.

The objective of the study was to evaluate the sperm viability of semen infected with PRRS viral particles and the effect of the Virus on the mobility of boar spermatozoa.

Material and methods

Animals:

5 stallions were used 19 months of age on average. The ejaculates were obtained by the gloved hand technique and processed commercial doses (BECZAR Insemination Center, Santa Rosa, Tecamachalco, Puebla, Mexico) with a concentration of 4×10^9 spermatozoa in 100 ml per dose of semen. We used a long-term diluent for samples, VITASEM LD (MAGAPOR®).

Before starting the study:

Of the 5 stallions sample took some semen and blood, to confirm they are negative PRRS virus using the technique (ELISA) and Real Time PCR.

The concentrations used for each dose of semen were $0, 1 \times 10^2$, 1×10^4, and 1×10^6 virus particles and each dose was tested in triplicate.

At each dose containing 1×10^6 spermatozoa was applied PRRS virus strain ATCC-VR-2332 (0, 102, 104 and 106 RNA copies/mL) was observed and recorded daily using the system parameters Analysis Computer Aided Sperm (CASA), Semen Analyzer (Hamilton Thorne®).

Variables assessed:

Curvilinear velocity, velocity rectilinear linear velocity, linearity, straightness index, lateral displacement amplitude, frequency mixing, moving cells, cells with progressive movement.

Statistical Analysis:

The different changes in sperm motility parameters throughout the day and between different concentrations of virus were analyzed with a two-way ANOVA in which the day were considered as blocks. The differences were analyzed post hoc Tuckey. $P < 0.05$ was considered significant. The analysis was performed using R 9.8.2 on a MacBook with MacOSX 10.6.

Results and discussion

Significant differences were observed with respect to the number of cells with total mobility in different days of evaluation. 201 ± 7.3, 167 ± 10.1, 165 ± 14.6, 134 ± 8.2 and 120 ± 8.8 for days 1, 3, 5, 7 and 10, respectively. While no differences between control and virus concentrations with respect to the percentage of progressive motility. It is also noted that there is a correlation (R = 0.8214) (P = 0.000) between the rectilinear speed (VSL) and curvilinear velocity (VCL) per 100. It was also noted that there is a correlation (R = 0.7209) (P = 0.000) between the curvilinear velocity (VCL) and amplitude of displacement (ALH) that made the heads in their curvilinear path from one side to another of its trajectory linear medium. Linear velocity (VAP) has a correlation (R = 0.7177) (P = 0.000) with the amplitude of lateral displacement (ALH), which means it has a direct correlation to the length of the displacement path of the sperm in their linear. This is the offset that effect their heads in a curvilinear path fro half its linear trajectory. We also observed a correlation (R = 0.6124) (P = 0.000) between the rectilinear speed (VSL) and displacement amplitude (ALH) that made the heads in their curvilinear path from one side to another of his middle path linear. Likewise there is a correlation (R = −0.6125) (P = 0.000) between Righteousness Index (STR) and the amplitude of lateral displacement (ALH). That perform their heads in a curvilinear path fro half its linear trajectory. Also note that there is a correlation (R = −0.6223) (P = 0.001) between the curvilinear velocity (VCL) and straightness index (STR) (%) is the percentage ratio between the rectilinear velocity (VSL) and linear velocity (VAP) by 100. Just as there is a correlation (R = −0.5389) (P = 0.000) between Rectilinear speed (VAP) and the Index Straightness (STR) (%) is the ratio between the velocity straight line (VSL) and the linear velocity (VAP) 100. Similarly we see that there is a correlation (R = 0.9015) (P = 0.000) between Righteousness Index (STR) with Linearity (LIN) is the ratio between the speed and the speed rectilinear VSL VCL for curvilinear 100).

Conclusions

The presence of different concentrations of PRRSV if the boar semen induces changes in the different types of sperm mobility.

Ejaculates infection with PRRS virus does affect sperm motility on days 1, 3, 5, 7.10 post infection. The correlation matrix showed that there were positive correlations with respect to the various parameters of CASA System, indicating that these indices altered sperm motility (VCL, VSL, VAP) suggest fertility problems or failure of sperm to reach the oocyte. Finally, we found a relationship between different types of derivatives house sperm motility, resulting in the addition of PRRS virus in boar semen affect sperm motility.

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Prebiotics and Probiotics in the Diet of Pigs from Weaning Termination and Its Effect on Behavior in Production and Quality Canal

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Summary: Crossbred pigs were used from 21 to 150 days of age (LM 100 × Pietrain) of both sexes, between 5.92 – 6.05 kg/body weight. They were divided into four treatments: T1 without inclusion; Prebiotics T2 4 kg/ton of feed (Saccharomyces cerevisiae) Probiotics T3 0.8 kg/ton feed (Lactobacillus spp, Bifidobacterium spp). T4 + Probiotics and Prebiotics 4 kg + 0.8 kg/t feed respectively (Saccharomyces cerevisiae) + (Lactobacillus spp, Bifidobacterium spp, (additive effect). T3 and T4 consumed 253.74 and 249.65 kg/food, the average for the four treatments was 250.86 kg the additive effect of T4 ($P < 0.05$) compared to other treatments, with a feed conversion of 2.49 kg of feed consumed per 1 kg of body weight gained, T2 and T3 were 2.51 and 2.50 kg of food consumed per 1 kg of body weight gained, and T1 had lower achievement.'s greater weight gain presented T4 with 101.79 kg, an increase of 3.82% compared to T1, with a final weight of 97.90 kg. for T1, T2, and T3 and T4, there were 5, 2, 2 and 0 respectively. Regarding the channel quality and behavior of the meat, the control had the better performance compared to the other treatments. was concluded that the addition of the prebiotics and probiotics had no positive effect. Economically greater benefit is obtained by using (4 kg/t of prebiotics + 0.800 kg/t of probiotics in food), which has a marginal return of 7.5:1.

Introduction

The pork industry in Mexico in recent years has achieved high levels of efficiency and profitability, which has placed within the major livestock industry nationwide, generating 350,000 direct jobs and 1.7 million indirect jobs, and contributing 1.7% to GDP (LXI Legislature Gazette, 2009). These indicators have encouraged domestic production of pork intensify and increase in parallel with population growth. In recent years researchers have been given the task of searching techniques that help improve production parameters, looking for alternatives such as the use of growth promoters and prebiotics and prebiotics also included in the diets (Czech, 2008).

This study is based on research and analysis of the use of prebiotics and prebiotics, which administered in the diets of pigs, which are offered from weaning until the end, encourage the growth and development of the intestinal flora. In addition to the effect of competitive exclusion decreases the number of pathogenic bacteria, by not being able to establish in the intestinal mucosa, for this reason if additional prebiotics and prebiotics in the diet of pigs, improve weight gain, feed conversion, performance and carcass quality (Figueroa et al. 2006).

The purpose of this research is to evaluate the diets supplemented with prebiotics and prebiotics to improve digestion and palatability of food, thus achieving optimal performance in the productive performance of pigs from 21 to 150 days of age and establish performance meat and

carcass quality.

Material and methods

For this experiment we used hybrid pigs commercial genetic lines(LM 100 × Pietrain) of both sexes, weaned at 21 days of age, with a weight range between 5.92 to 6.05 kg liveweight. They were divided into four treatments: T1 (n = 100) Without inclusion, T2 (n = 100) 4 kg of Prebiotics/ton of feed (Saccharomyces cerevisiae), T3 (n = 100) Probiotics 0.8 kg/ton feed (Lactobacillus spp, Bifidobacterium spp). and T4(n = 100) Prebiotics + Probiotics 4 kg + 0.8 kg/t feed respectively(Saccharomyces cerevisiae) + (Lactobacillus spp, Bifidobacterium spp, (additive effect). then were housed in facilities according to their growth phase (Stages weaning, growth, development and completion) where each pig was weighed and recorded their data in the analysis of the study. Finally, once slaughtered pigs were evaluated in muscle development characteristics, making cuts and measuring the different pieces of meat pigs subjected to each experimental treatment. was used completely randomized design, were used for this experiment 400 pigs weaned 21 days of age, randomly assigned, according to the group corresponding littermates, production variables assessed food intake, weight gain, weight gain, feed conversion, mortality rate and carcass.

Statistical Analysis

We used ANOVA and Tukey statistical test to compare means. The evaluation of the channel, basic descriptive statistics were used, mean and standard error. All results were processed using the statistical software package SPSS® V15.

Results and discussion

Food consumption

It is observed feed intake per treatment, there was no difference ($P > 0.05$) among treatments. By adding thereto prebiotics and probiotics to determine the additive effect, these intakes were similar, the T4 was 249.65 kg of food, and there is a 0.5% decrease in food intake compared with the average consumption of the four treatments(250.86 pounds of feed), T1 was 250.13 kg. For T2 was 249.92 kg and 253.74 kg T3 was. Other researchers demonstrated decreases from 0.5 to 1% in feed intake compared with consumption of the control treatment(Guarner, 2000 and Bocco et al 2002), which is consistent with the findings in this study.

Feed conversion

By using growth promoters based prebiotics, probiotics and the additive effect between the two, it is observed that T4 was significant($P < 0.05$) compared to other treatments, where T4 had a feed conversion of 2.49 kg feed consumed per 1 kg of body weight gained to the T2 was 2.51 kg, 2.50 kg T3, T1 and had a lower performance compared to the other treatments.

Other studies show that the relationship between prebiotics and probiotics, will perform better, improving significantly the consumption and feed conversion, but to date no more studies that support these findings(Cagigas and White, 2002).

Cattle weight

The ANOVA found highly significant difference ($P > 0.05$) among treatments. The higher weight gain was presented with 95,874 treatment 4 kg of body weight from baseline, which was 21 days of age to 150 days of age, this represents an increase of 4.13% compared to treatment 1. On the other hand, treatment 2 had a mean weight gain of 92,941 kg bodyweight cattle Treatment 3 presented an average weight gain of 93, 168 kg bodyweight livestock, treatment 1 had gained weight average of 91,916 kg These data are consistent with other researchers(Quiles and Hevia, 2005).

Weight gain

The comparison test of Tukey shows that treatment 4 ($P > 0.05$) was statistically superior to the other treatments. Treatments 2 and 3 are statistically similar to each other, so no one treatment which had a lower weight gain compared to treatments where they added a growth promoter(Chiquieri et al., 2006).

Mortality rate

Regarding the incidence of mortality, there were 9 cases in the four treatments, being in treatment 1 (control), five dead, while in T2, T3 and T4, were 2, 2 and 0 respectively. The reduction of losses in the experiment indicate that adding prebiotics, probiotics and prebiotics the additive effect and prebiotics in the pig feed, was reduced by 100% mortality cases compared to the control treatment. It is shown that a positive relationship exists regarding mortality decreased with the addition of a growth promoter basis of prebiotics and probiotics in food for pigs.

Economic evaluation

We determined the rate of return on investment (Troi), T4, presents the greatest economic benefits, followed by T1 and T2, showed the lowest economic benefits is the T3. The T4, has a rate of return on investment of 7.5:1 on T1. If using the T4, compared to Q1, is recovered for every dollar($ 1.00) invested an additional $ 7.50. Valdez(2007) observed that piglets fed with growth promoters based Prebiotics, probiotics and enzymes, have greater economic benefits, unlike control group piglets which were lower economic benefits derived from the sale foot of piglets. The marginal rate of return for treatments with growth promoters based prebiotics, probiotics and enzymes, showed a 10% to the

investment.

Conclusions

Weight gain showed that the hypothesis, is not rejected, making a difference of 3.89 kg higher compared to control treatment. It reduced the risk of post-weaning mortality, in Q4 by 100%, by the additive effect (Prebiotics + Probiotics). A decrease in food consumption by 0.5% compared to other treatments, which reduces production costs. In Q4, we obtained a feed conversion of 2.49 kg vs 2.54 ($P < 0.05$) of food consumed by the control group, at the rate of 1 kg of body weight gained by improving this parameter compared to other treatments. Regarding the quality of the carcass and meat behavior, the control had the better performance compared to the other treatments. Economically greater benefit is obtained by using(4 kg/t of prebiotics + 0.800 kg/t of probiotics in food), which has a marginal return of 7.5:1.

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Factors That Cause Reproductive Disorders and Low Fertility in Cows

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Summary: At present, it is necessary to produce more milk per cow more efficiently, because the undeniable improvements in the dairy industry, in terms of nutrition and food, mainly in formulating rations for animals, according to the levels production, as high, medium and low, and in herd genetics. However, these improvements have resulted in problems, the results of which is usually the presence of reproductive problems, generally resulting in low reproductive and productive performance of the animals. Despite the advances that exist today on feasible technologies applied in breeding and animal production, few farmers and ranchers have shown concern for improving the general environmental conditions of animal production units, without taking into account that these conditions are largely responsible for animal health, welfare altering and impacting them significantly in the presence of problems of a reproductive and productive, significantly increasing production costs in the dairy industry. Describes some important factors that cause reproductive disorders and low fertility in cows.

Introduction

During the last two decades, advances in genetic improvement, the area of nutrition and improving the management of the animals, have made production averaged specialized cows milk production is increasing, without however, the increase in milk production, has had a negative impact on reproductive performance of these animals, such as low conception rates, which do not correspond to those managed for 20 – 30 years so that before could achieve conception rates of 60%, at present and in the best case, can only be achieved from 40% to 50%. From all this, we can say that today, you can accept the 15% – 16% of cows in estrus repetitions problems, which means achieving at least 45% of conceptions (Cavazos, 2001; Córdova et al. 2002).

Material and methods

A review of the factors that can cause reproductive disorders in cattle.

Results and discussion

Are some critical points related to the problem of fertility in dairy cows.

Conclusions

Factors that may cause reproductive disorders and low fertility in cows are multifactorial.

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Preruminant Lamb's Diarrhoeas: Treatment by Ecological Pure Medicine

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Introduction

It's well known that basic feed digestion in newborn ruminants takes place in the abomasum and small intestine. Technological stresses during the first weeks of life provoke dysfunction of parietal glands in abomasal lumen resulting in achlorhydria. The natural barrier against environmental micro flora destroyed and animals get sick with symptoms of dysbacteriosis. The latter results in diarrhoea and low body weight gain. The similar processes were shown in preruminant calves [1]. To prevent diarrhoea and its consequences we decided to stimulate HCl secretion with ecologically pure preparation sodium acetate (Khimprom JS). Similar methods of rearing newborn calves [2] and weaned piglets [3] are wide spread in Russia.

Materials and methods

Trials have been conducted in "Khanata" farm (Kalmyk Republic) during the period of mass lambing. 60 newborn diarrheic lambs of Soviet Merinos breed were divided into two groups (experimental [EG] and control [CG]) and given either 3% sodium acetate aqueous solution [SAAS] or saline. All medicines (5.0 ml) were administrated orally every morning by the syringe cannula in 7 days. Animals were kept in neighbor pens with their dams according to traditional Kamykian standard. Body weight (BW) of lambs was measured before and after treating. Results are expressed as the means S. D. Statistical analysis was performed using Student's t test. Differences between paired values were considered significant at $P < 0.05$.

Results and discussion

Animals of EG were ill approximately 3 time less, and gained 32.36% more against the lambs of CG (all values significant). We think that these beneficiary effects are due to stimulation of the abomasum parietal glands.

Table 1 Results of 3% SAAS applying

Group	Days of illness	BW before treating (kg)	BW after treating (kg)	BW gain
EG	1.3 ± 0.2	5.50 ± 0.42	9.49 ± 1.33	3.09 ± 0.65
CG	3.8 ± 1.1	6.01 ± 0.41	8.90 ± 1.76	2.09 ± 0.87

Physiological experiments on operated animals showed acetic acid (as well as 3% SAAS) to be a dramatic stimulant of abomasal HCl secretion in adult sheep [4] and preruminant calves [5]. Moreover, as we showed before in diarrheic milk-fed calves, pH of abomasal juice did not grow after feeding in the trials with the infusion of 3% SAAS. In contrast, it grew 4.6 times in the control trials (with saline infusion) [6].

Recent progress in the field of energy homeostasis was triggered by the discovery of the hormone leptin and revealed a complex regulatory neuroendocrine network. A late addition is the novel stomach hormone ghrelin, which is the motilin related family of regulatory peptides. In addition ghrelin stimulates appetite and induces positive energy balance leading to body weight gain [7]. Many of ghrelin influences as well as vagal effects are similar to the action of acetate contained preparations. These facts made us to assume hypothesis that 3% SAAS secretagogue phenomenon involves above mentioned neuroendocrine complex.

Conclusion

3% SAAS might be recommended in practical using in rearing lambs as its beneficiary influence on animal welfare.

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Indicators of Lead Exposure in Cattle and Sheep

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Summary: The aim of this work was to study the significance of blood lead, zinc-protoporphyrins and hair lead concentrations as a parameters of lead exposure in cattle and in sheep. Two heiffers FFPN and a group of five ewes received lead orally at a dose of 2. 3 mg/kg/d for 17 weeks and 62 days respectively in cattle and ewes. In addition a control farm and five farms in a polluted area were used; in each farm fives cattles were chosen randomly. In cattle blood lead concentrations reached 360 μg/l threshold from which the manifestations of toxicity may occur, whereas it reached 122 μg/l in ewes. After the end of exposure the blood lead levels decreased in a biexponential-like manner and were higher than the measured before the exposure period. The lead concentrations were 0. 70 ± 0. 3 mg/kg in first sampling and 0. 9 ± 0. 2 mg/kg four months later in hair bovine located in rural area. Besides, hair lead levels were higher in urban area, from 4. 2 ± 1. 2 mg/kg (first sampling) and 13 ± 3. 5 mg/kg (four months later) which suggest that the animals were under influence of anthropogenic activities.
Key words: lead, exposure, blood, hair, zinc-propotoprphyrins, cattle, ewes.

Introduction

In spite the decrease of lead contamination following the using of fuel without lead through the world, it still a hazard to the environment and poses a threat to human and animals health as a consequence of low doses ingestion for a long period. Lead is a metal not biodegradable and accumulate in environmental compartments and in organisms. Lead analysis would established its presence in differents compartments of an ecosystem. Many species (flora and fauna) are used as a bioindicatrors of lead contamination since they can reveal the quality of the environmental area where they are living and to assess habitat status.

The ingestion of metal through water and food can lead to toxicity. Lead toxicity has been reported to be related to impairements of chlidren's mental development and learning capacity, and in response to this in 1987 experts at the FAO and WHO set the provisional tolerable weekly inake at 25 μg/week/kg body weight (WHO, 1987). Besides biochemical modifications even at low doses have been reported in humans (Sakai, 2000) and animals (Mehennaoui and al, 1988; Liu, 2003)). In human, traditionnally metals levels in blood and urine have been used for biological monitoring of exposure and risk (Skerfving and Nilsson, 1992). In addition several sensible and specific tests are used to control exposed individus: Delta-Amino-Levulinic Acidic Deshydratase (ALAD), Lead Chronic exposure can be monitoring by measuring the levels of ALAU, ALAD and Zinc-Protoporphyrins in blood. (Poonam and Farhat Jaffery, 2005). Hair is an adequate biological sample to asses metal exposure in humans as well as in animals; hair is a non invasive method, easy to sample, and to conserve for a long period.

We have been conducted a study on cattle in the vicinity of lead and zinc ore-processing factory in northern France (Mehennaoui et al., 1988), a study on sheep which indicate the toxicokinetics of lead (Mehennaoui et al., 1997), and a study in cattle in north-east Algeria (Afri-Mehennaoui et al., 2001). In those studies, hair were used as indicator of lead exposure. Besides there was a relationship between blood lead levels and zinc-protoporphyrins levels.

The aim of this work is to present the principal results of those studies and to ascertain whether lead storage in hair was a significant indicator of exposure and to explain the toxicological significance of blood levels and zinc-protoporphyrins levels in both cattle and sheep.

Material and methods

In experimental station

Repeated exposure of two Heifers FFPN race at a dose of 2. 3 mg/kg/day for 17 weeks (lead caused by contaminated hay) followed by a 30 week recovry period. (Mehennaoui et al., 1988)

Repeated exposure of five ewes at a dose of 2. 3 mg/kg/day for 62 days (Lead chlorure in oral capsules)

followed by a recovry period of 81 days.

Establishment of a monitoring plan in two areas using catlle as sentinel species and the hair as an indicator of lead exposure

One control farm and five tests farms were included in this study. The control farm was located in a zone without influence of anthropogenic activities and qualified as uncontaminated area. Five adult cattle of about 4 years of age were randomly selected. The animals were fed hay and barley. The water was given ad libitum. The five farms were located along the wadi which cross the principal town of batna(North-east of Algeria). The wadi receives several contaminats from the industrial activities and the waste water. The pasture were irrigate with water coming from the wadi and contaminated the forage reserved to bovines. Five adults' cattle were randomly selected in each farm.

Sample collection

Blood samples were collected from the left jugular vein of cattle and ewes into special heparinised tubes under vacuum(ref. 367735 Vacutainer Tubes, Becton Dickinson, Mayland, France), known not to interfere with the analysis of trace elements, metals or metalloids.

In cattle: the collection was performed every week for 47 weeks.

In ewes: the collection was performed on the morning before oral administration of the inorganic metallic salts on days 7, 14, 21, 24, 28, 31, 35, 38, 42, 45, 49, 51, 52, 56, 59, 63, 66, 70, 77, 91, 105, 119 and 133. Other blood samples were also collected into heparinised tubes in order to measure the zinc-protoporphyrins (ZPP) concentrations every two weeks on days 0, 14, 28,..., until day 98.

Hair collection: hair was only collected in cattle. Two hair samplings were done and placed in a polyethylene bag. Hair was clipped from the tail. The second hair sampling four months later was from hair which had grown again.

Chemical analysis

We measured the lead concentration in blood by electrothermal atomic absorption spectrophotometry with a 1100 B Perkin Elmer AGA 700 spectrophotometer, as described by Mehennaoui et al. (1997).

Hair lead concentration was determinated by electrothermal atomic spectrophotometer with an Analyst 100 Perkin Elemer as described by Afri-Mehennaoui et al. (2001).

Zinc-protoprphyrins was measured using an appropriate hematofluorometer(AVIV type) haemoglobin standard value of 15 g/100 ml.

Results and discussion

Fig. 1 and 2 shows the mean blood lead concentration-time curve for the time during and after repeated oral administration of lead respectively in cattle and ewes. In cattle: as soon as the administration of lead salt, lead levels in the blood increase very quickly reaching a plateau around 360 μg/l threshold from which the manifestations of toxicity may occur. After the end of exposure, the blood lead levels decrease first with a steep slope, then more slowly to values of prior treatment after 24 weeks.

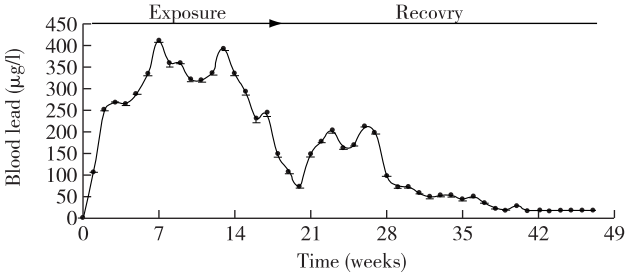

Fig. 1 Mean blood lead concentration in two cattles after oral administration of 2.3 mg/kg Pb for 17 weeks

In ewes: The mean blood lead concentration increased from the start of the exposure period to the second week of exposure when it reaches a plateau. During the plateau, the mean blood lead concentrations were 122 ± 36 μg/l. After the end of exposure the blood lead decreased in a biexponential-like manner. At the end of the sampling recovery period, the blood lead concentration was higher than that measured before the exposure period.

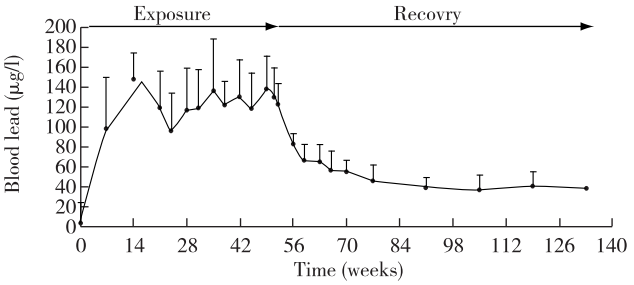

Fig. 2 Evolution of blood lead (means ± standard deviation) in fives ewes after lead repeated oral administration of 2.3 mg/kg/d for 52 days

Fig. 3 and 4 show the evolution of zinc-protoporphyrins levels during and after the exposure period. One of the metabolic effects of lead is the inhibition of heme synthetase resulting accumulation of protoporphyrins that combine zinc to form a complex ZPP endowed with a fluorescence(property used to put in evidence the presence of metabolically active lead.). As inhibition took place in the erythroblaste the effect is noticeable only after rejec-

tion of erythrocytes from the bone marrow into the bloodstream. This lagtime corresponds to the life of the red blood cell (about 2 months in ruminants). The levels of ZPP in cattle non exposed are of 7 μg/100 ml; in ewes non exposed they are relatively stable and vary between 12 and 16 μg/100 ml. In treated animals the level of ZPP increases as early as the fourth week and then stabilizes. The elevation of the ZPP in the sheep is more important and less forager than that observed in cattle treated with the same dosage. Blood lead concentration and ZPP concentrations are good indicators of lead exposure if one considers that blood lead concentration increases quickly stabilizes within one to two weeks and quickly decreases after the end of exposure while ZPP concentrations increase only after a delay of 1 or 2 months and remain high after the end of exposure.

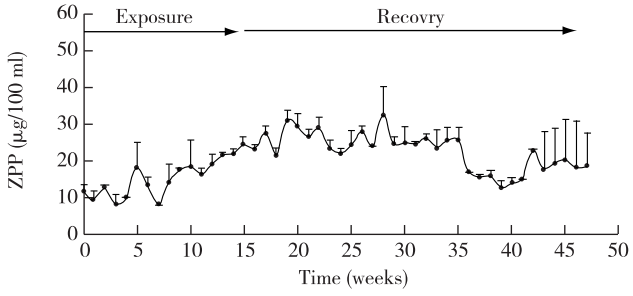

Fig. 3 Zinc-protoporhyrins evolution in 2 cattles after lead repeated oral administration of 2.3 mg/kg/d during 17 semaines

Hair lead concentrations in cattle are shown in Table 1. In control cows hair lead concentration was 0.70 ± 0.3 mg/kg for the first sampling and 0.9 ± 0.2 mg/kg for the second sampling. Concentrations in the second sampling were not significantly increased. We should be noted that the mean hair lead concentration in farm 5 was similar to that observed in control farm. In return, mean hair lead concentrations were higher in the other farms than that of control farm. The means values ranged from 2.70 ± 1.4 to 4.1 ± 1.9 mg/kg for the first sampling. In the second sampling the levels of lead hair were significantly increased for farm n° 2, farm n° 4 and were respectively 13 ± 10 and 4.5 ± 3 mg/kg. In test farms n° 1 and 3 the increasing level of hair lead in the second sampling were not significantly different and were 5.7 ± 3 and 2.9 ± 1.7 mg/kg respectively. Variations of hair lead concentrations in test animals (all samples) are high. Hair lead concentrations were increased for the second sampling in test animals (3 ± 2 versus 6 ± 4 mg/kg). The concentrations obtained in test animals were significantly different in various ways. Several studies were conducted by using hair as an indicator of trace metal exposure in public health (Wilhelm and al., 2002; Sanna and al., 2003; Strumylaite and al., 2004) and in ruminants (Mehennaoui and al., 1988; Ward and Savage, 1994; Khan and al., 1995).

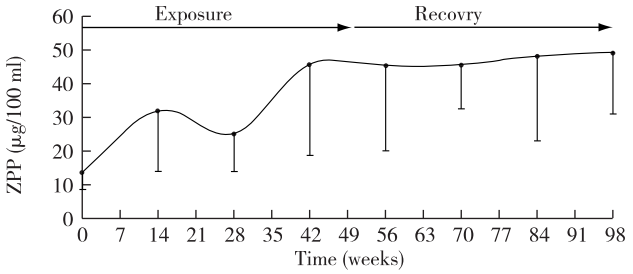

Fig. 4 Mean blood protoporphyrins concentration (mean ± SD) during and after the period of exposure

Table 1 Hair lead Concentrations (mean ± standard deviation) in control and test animals

Farms	First sampling	Second sampling	Test T-Student (probability)
Control (n = 5)	0.70 ± 0.3 *(a)	0.9 ± 0.2 (a)	P = 0.09
Farm 1 (n = 5)	4.13 ± 1.9 (b)	5.7 ± 3 (b)	P = 0.27
Farm 2 (n = 5)	3.7 ± 2.3 *(b)	1.3 ± 0.10 *(b)	P = 0.05
Farm 3 (n = 5)	2.7 ± 3.2 (a)	2.9 ± 1.7 (b)	P = 0.46
Farm 4 (n = 5)	2.7 ± 1.4 (b)	4.5 ± 3 (b)	P = 0.048
Farm 5 (n = 5)	0.9 ± 0.4 (a)	1.2 ± 0.6 (a)	P = 0.18
All samples (n = 25) (Test animals)	3 ± 2 *	6 ± 4	P = 0.055

The mean values, standard deviation and statistical significance are reported.
The same letters in the same column indicate the difference was not significantly different.
* $P < 0.05$: between Control and test animals (all samples)

The lead concentrations in hair were about 10 times higher than those measured in animals living in uncontaminated area. The level of lead in hair should be revealed the amount ingested by the animals during its genesis. The extent and the time of exposure were the most important factors which had an influence on the lead hair concentration. Higher the exposure, higher the lead quantities excreted in hair. Our results indicate that the

levels of hair lead are two time lower than those reported by Mehennaoui and al. (1988) in cattle exposed to lead and zinc ore-processing, about 9 mg/kg. It suggests that the levels of hair lead depend on the amount ingested by animals and on the extent of exposure. The levels of lead in hair of control animal reveal the background level. It is necessary to determine the background level in every country because it varied from one to another region through the world. In Algeria, there is a lack of data about the level of lead in biological samples of cattle. Therefore our investigation give information's about the level of lead contamination in the study of interesting. The increasing level of lead in test hair cattle may be do the ingestion of soil or contaminated forage. Besides ingestion via contaminated soil, feed and water are the major routes for Pb uptake for cattle while inhalation can be neglected. Hair samples are appropriate to evaluate the lead level of exposure and should be considered as an epidemiological indicator

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Some Bacterial Causes of Pneumonia in Camel Calves AT Matrouh Governorate

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Summary: The current work was conducted in three regions at Matrouh governorate, Egypt, 2012. The work included 50 camel calves of 1 – 10 months old, both sexes and showing the clinical signs of pneumonia. The animals were divided into two groups according to their ages, young(1 – 6 months) and old(7 – 10 months). From the total number of animals, 5 cases were slaughtered, 35 died and 10 treated. Swabs for bacteriological examination were collected from upper air passages and affected lung tissues. Bacteriological examination revealed that Gram-negative microorganisms were the most prevalent(52%) as compared with the isolated Gram-positive bacteria(48%). *E. coli* represented the highest percent of incidence(20%), while *Pasteurella multocida* was at the lowest value(4%). Various incidences were reported among the other isolated strains. The clinically diseased group responded to the planned treatment.
Key words: Bacterial flora, camel, epidemiology, pneumonia

Introduction

Pneumonia is a major disease problem of domestic animals. Outbreaks occur in camel as well as other animal's worldwide (Al-Doughaym et al, 1999). Pneumonia may be caused by bacteria, viruses, parasites and fungi (Schwartz and Dioli, 1992; and Al-Ani, 2004). The economic losses due to pneumonia in camels are represented by loss weight, losses due to condemnations during meat inspection and mortalities(Al-Ani, 1990, and Mohamed et al, 1990). In Egypt, Farrag et al. (1953) reported the isolation of eight genera of bacteria from camel pneumonic lungs. Mahmoud et al. (1988) reported a variety of microorganisms from pneumonic lungs of camel. *Escherichia coli*, *Staphylococcus* spp., *Klebsiella* spp., *Pseudomonas aeruginosa*, *Actinomyces pyogenes*, and *Streptococcus* spp., are the most prevalent causes of camel pneumonia (Al-Doughaym et al, 1999; Al-Rawashdeh et al, 2000; and Al- Tarazi, 2001). Some workers studied camel pneumonia in Sudan and other parts of the world(Khan, 1970; McGrane and Higgins, 1985; Mustafa, 1992; Pavlik, et al., 2002; and Nasr, 2003). Their studies stated the seasonality in the occurrence of camel pneumonia and established the many etiological agents responsible for the condition such as *Staphyolcoccus* spp., *Corynebacterium*. spp., *Streptococcus* spp., *Klebsiella pneumonae*, *Diplococcus pneumoniae*, *E. coli*, *Bacillus* spp., *Pasteurella* spp., *Haemophilus somnus Micrococcus* spp., *Actinomyces* spp. and *Mycobacterium* spp. The current study was conducted to isolate and identify the types of bacterial species involved in pulmonary lesions of camel and its association with pneumonia.

Material and methods

During the last year, the veterinarian at Matroh found that there were varying degrees of respiratory affection among camel flocks. The total number of animals included was 50 of different ages, sexes and belonged to 3 localities in Matrouh governorate, Egypt. The work was done during 2012. Clinical observations and examination revealed that 50 animals showed pneumonia. The animals were divided into two groups according their ages, young(1 – 6 months) and old(7 – 10 months). From the total number of animals, 5 cases were emergency slaughtered, 35 cases died and 10 treated. Gentamycin, electrolytes and dexamethazone were the drugs used in treatment. These animals were grouped as follow:

Table 1 Divisions, ages, and numbers of animals used

Age(months)	No. of camel calves	Diseased	Slaughtered	Dead	Treated
Young age(1 – 6)	30	30		30	
Old age(7 – 10)	20	20	5	5	10
Total No. of animals	50	50	5	35	10

The lungs were examined by visual examination and palpation for the presence of post mortem lesions; after

which samples for bacteriological investigations were collected from both normal and abnormal lungs. Samples for bacteriology were placed in polythene bags and kept in flask containing ice and taken to the laboratory. (Barrow and Feltham, 1993). Swabs from the nose, larynx from diseased animals and samples from the affected lungs tissues from emergency slaughtered and dead animal were collected in clean plastic bags. During collection it was noted that nasal and laryngeal swabs were taken under complete aseptic conditions. Lungs of slaughtered and dead animals showing pathological changes were taken in plastic bags, placed in sterile jars. The surface of affected organs was seared with hot spatula then opened with sterile scalpel. The pus materials was taken with a sterile platinum loop and inoculated in the culture media for bacterial examination. Samples were collected immediately after evisceration, transferred to laboratory at the research institute, Matrouh province for direct microscopic examination, Isolation and identification of microorganisms. The bacterial examination by inoculating the collected pus or lung tissues onto blood agar and (MacConkey) agar plates then the plates incubated at 37℃ for 24 – 48 hours. Suspected colonies were subjected for complete identification. Isolates obtained were identified morphologically, culturally and biochemically according to Cruickshank(1975).

Results

The results of the present work were recorded in the following tables:

Table 2 Bacterial species isolated from affected lungs of 50 camels

Organisms	No. of strains	Percentage
E. coli	10	20%
Streptococcus pneumoniae	4	8%
Pasteurella multocida	2	4%
Cornyebacterium pyogenes	8	16%
Staphylococcus epidermidis	5	10%
Staphylococcus aureus	9	18%
Pseudomonas aeruginosa	5	10%
Klebsiella pneumoniae	7	14%
Total	50	100%

Table 3 Isolated bacterial strains of different groups

No. of cases	Age and No.	*E. coli* 10	*Strept. pneumoniae* 4	*P. multocida* 2	*Coryn. pyogenes* 8	*Staph. epidermidis* 5	*Staph. aureus* 9	*Pseudo. aeuroginosa* 5	*K. pneumonae* 7
Diseased cases 50	Young age(30)	8	2	-	6	2	5	4	3
	Old age(20)	2	2	2	2	3	4	1	4
Slaughtered cases 5	Young(–)	–	–	–	–	–	–	–	–
	Old(5)	–	2	–	1	–	1	–	1
Dead cases 35	Young(30)	8	2	–	6	2	5	4	3
	Old(5)	1	–	1	–	–	1	1	1
Treated cases(10)	Young(–)	–	–	–	–	–	–	–	–
	Old(10)	1	–	1	1	3	2	–	2

Discussion

The remote breeding system of camels in Egypt, considered to be one of the major constrains that facing diagnosis of camels diseases. Our observations denote that the disease was severe and danger in young camel calves as compared with the old ones. Bacteriological findings of our study were reported in Table 2 and 3. *E. coli* was the most prevalent organisms(20%) isolated from pneumonic camel calves. Theses results were similar to the findings of Mahmoud et al. (1988) and Mustafa, (1992). The high isolation rate of *E. coli* correlates with the natural habitat of *E. coli*, where it can survive in fecal particles, dust and water for weeks and months (Quinn et al., 1999). The incidence of *Staph. Epidermidis and Staph. aureus* organisms(Table 2) were

10% and 18% respectively. The organisms found incriminated in pneumonic lesions were varied in severity and often isolated in mixed cultures with other bacteria. Nasr(2003) isolated *Staphylococcus* spp. from upper and lower respiratory tract of camels showing pneumonic signs at anti and postmortem examinations. *Corynbacterium pyogenes* and *Streptococcus pneumoniae* (Table 2 and 3) were among the second important pathogens involved in camel's lung infection mainly in the pyogenic lesions. This agreed with the results obtained by (Mustafa 1992, M. S. Abubakar, 2010 et al.). *Corybacterium pyogenes* were found representing (16%) of the total bacterial isolates. The organism was isolated from pyogenic lesions, grey hepatization and adhesion. In addition to *Escherichia coli* two other Gram-negative isolates were identified. Theses were *Pseudomonas aeruginosa* (10%) and *Klebsiella pneumoniae* (14%). The results were similar to that of (Nasr, 2003. Zubair et al., 2004; El-Tigani et al., 2004; Kane et al., 2005). Their results confirmed the presence of the organisms as upper respiratory tract flora and they may invade the lungs when the immunity of animals decline. *Pasteurella multocida* represents the lowest incidence (4%) of bacterial infection among pneumonic camel calves. The organism was isolated from congested lung lesions showed haemorrhage and inflammation of the lungs. Isolation of the organism was previously reported by Mustafa(1992).

The general treatment of pneumonia often requires antimicrobial therapy. The decision of antimicrobial therapy depends largely on the sensitivity of the targeted microorganisms and the pharmacokinetics of the drug to achieve the desired therapeutic concentration.

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Comparative Animal Hygiene Assessment of two Technologies for Buffalo Manure Processing

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Summary: The Organic crop production is one of the main pillars of development of the agriculture in Bulgaria. Regulation No. 22/2004 of the MZG offers the opportunity to improve soil fertility through the use of organic fertilizers, which are processed by fermentation or composting. The purpose of this paper is to explore the use of two regulated in Ordinance No. 22 byproducts of the processing of fertilizer-compost, obtained after methane fermentation, and composting. After laboratory experiments and mathematical modeling, the optimal technological parameters were found to be: dry matter content of 7%, mesophilic temperature range of 330 Celsius and time of degradation of organic matter 49% –51% for 15 days. An assessment is made of the qualities of the methane fermentation byproduct (compost) and compost by physiochemical parameters, nutrient content of macro and micronutrients, and opportunities to use it to improve soil fertility.

Introduction

The reason for the processing of manure is that it poses threat to the biocenosis due to the presence of pathogens, helminth eggs, viruses and fungi. The Regulation 22/2004 provides two options: methane fermentation or composting. During the last few years research on the technologies for manure processing in biogas installations was increased. The purpose of this paper is to explore the use of the two regulated in Ordinance No. 22 byproducts of processing of fertilizer: compost-obtained after methane fermentation, and compost. This study establishes criteria of the status of raw material for the amount of basic biogenic elements and their content in both products: bioslime and compost.

Material and methods

According to Regulation No. 22/2004 manure was used from farm for breeding with extensive bezpostelno breeding of bulls. Buffalo manure was treated with water and homogenized until obtaining a suspension with 7% solids. On input of the system organic, in the processes of digestion the following analysis were conducted:

– Determining of pH – by electronic pH meter OP – 211/1 radelkis – Budapest.

– Determining of total dry residue – total residue after ignition and loss on ignition – BDS 17. 1. 4. 04-80(T 58). Preserving of nature. Hydrosphere. Indicators of water quality. Methods for determination of total dry residue, suspended and dissolved solids.

– Determining of ammonium nitrogen – Method fotokolorimetrichen Nessler – BDS 17. 1. 4. 10-79(T 58). Preserving of nature. Hydrosphere. Indicators of water quality. Methods for determination of ammonia, S., 1980.

– Determining the concentrations of methane and carbon dioxide by gas analyzer include electronic company Dräger.

Experimental research at sequence-batch process of methane fermentation have been carried out in a bioreactor with 2 L capacity and working volume of 1 L designed in the Institute of Microbiology in BAS. Methane fermentation was carried out at temperature 32 ± 1 ℃, 7. 2 at homogenization of the substrate for 1min every hour.

Quality assessment of the products from methane fermentation and composting according to physicochemical indexes, including biogenic macro- and microelements quantity and possibilities for the use for increasing soil fertility. The same analyses were carried out on the processed organic matter as on the input substrate. A validated intra-laboratory method RESID/02/04 and intra-laboratory methods BDS-EN 13342, BDS EN-13346 were used. pH, organic matter and dry matter - ILM; Nitrogen determination - BDS-EN 13342. Determination of P_2K_5, K_2O, CaO, MgO, RESID/02/04. Determination of $N\text{-}NH_4$, $N\text{-}NO_3$, P_2O_5, K_2O, $S\text{-}SO_4$, ILM

Results and discussion

Assessment of the quality of the methane fermentation product (bioslime) by nutrient content of macro-and micronutrients and opportunities to use it in soil fertility enhancement.

After methane fermentation the ration methane and CO_2 is 70:30.

Data on nutrient content of macro-and micronutrients are presented in Fig. 1 to 4.

After continuous process of digestion of buffalo dung, the total quantities of macroelements, calculated on the dry matter-N, P_2O_5, K_2O, CaO will be increased

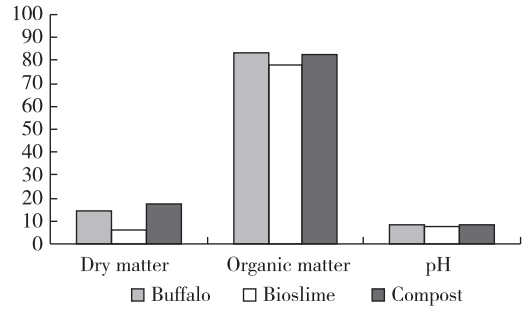


Fig. 1 Dry and organic matter

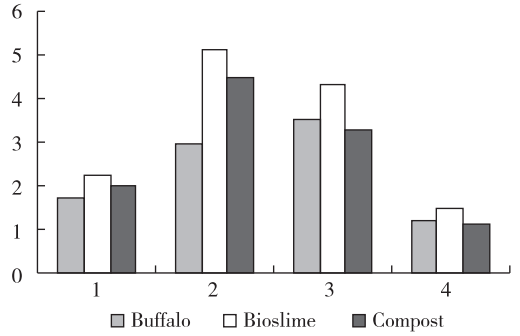
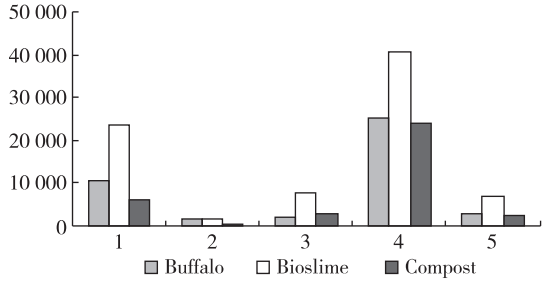


Fig. 2 Content of P_2O_5, K_2O, CaO, MgO



Legend: 1 - N-NH_4; 2 - N-NO_3; 3 - P_2O_5; 4 - K_2O; 5. S-SO_4

Fig. 3

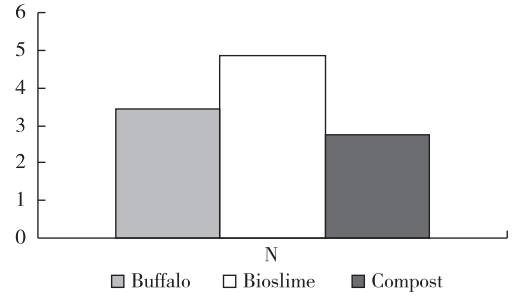


Fig. 4

in different degrees compared with compost inputs. N increased by 42.56% from 3.74% ±0.14% (input) of 4.99% ± 0.19% in the compost. P_2O_5 increased by 31.40 percent in the boslime of 2.43% ± 0.06% and 1.81% ± 0.04% input material. K_2O from 2.74% ± 0.74% increased by 71.46% to 5.00% ± 0.51% bioslime. CaO increased by 23.85% in the bioslime from 3.70% ±0.35% to 4.60% ±0.12%. The concentration of MgO increased by 22.52% in the bioslime (from 1.09% ±0.12% to 1.37% ±0.05%).

Similar are the results and the content of water-soluble fractions of macronutrients, calculated on the dry matter. For example, N-NH_4 from 10214 mg/kg in the input material, increased to 23256 mg/kg, (125%) in bioslime and N-NO_3 decreased by 17.85% – in bioslime – 1259 mg/kg in comparison with input material – 1551 mg/kg.

The content of P_2O_5 from 2024 mg/kg increases by 280.8% in the bioslime to 7603 mg/kg.

The content of K_2O from 25398 mg/kg increases in bioslime with 61.88% to 41001 mg/kg.

The content of S-SO_4 from 2674 mg/kg increases in bioslime with 151.03% to 6766 mg/kg.

Buffalo manure composting:

Composting is an aerobic process in natural or artificial aeration. It is divided into four stages: mesophilic, thermophilic, cooling and maturation. The composition of compost is widely (% of dry weight): organic matter 25% – 80%; C 8% – 50%; N 0.4% – 3.5%; P 0.1% to 1.6%; K 0.4% to 1.6%; Ca 0.7% to 1.5%. In composting all pathogens, including spore-forming microorganisms die in a short time.

In the process of composting buffalo manure the total quantities of macroelements, calculated on the dry matter-N, P_2O_5, K_2O, CaO change to varying degrees in the output material (compost) in relation to inputs. Thus N is reduced with 19.56% from 3.34% ± 0.14% (input) to 2.56% ±0.11% in the compost.

P_2O_5 increased by 17.95% in the compost of 1.69% ±0.03% from 1.39% ±0.04% at the entrance.

K_2O from 2.54% ±0.74% increased by 53.04% to 4.87% ±0.46% in the compost.

CaO is reduced by 6.47% in the compost from 3.70% ±0.35% to 3.37% ±0.33%.

MgO concentration is reduced by 8.51% in the compost (from 1.22% ±0.12% to 1.09% ±0.11%).

Thats not the situation with the water-soluble mineral nutrients, calculated on percentage of the dry matter. For example, N-NH_4 from 10514 mg/kg in the input material in the compost decreased by 41.76% at 6185 mg/kg and

$N\text{-}NO_3$ in the compost decreased by 74.92% – 387 mg/kg compared to incoming raw materials – 1551 mg/kg.

P_2O_5 content of 2000mg/kg increased by 27.55% in the compost to 2551 mg/kg.

The content of K_2O from 25198 mg/kg reduced by 4.82% in the compost to 23929 mg/kg.

The content of $S\text{-}SO_4$ from 2634 mg/kg is reduced with 19.04% in the compost to 2151 mg/kg.

Comparing bioslime and compost we notice that the total quantities of macroelements calculated considering dry matter - N, P_2O_5, K_2O, CaO are higher in bioslime than in compost.

N in bioslim is 4.67% ±0.19% which is 76.04% higher – compared to the amount of compost, which is 2.56% ±0.11%.

P_2O_5 in bioslime is 2.31% ± 0.06% which is 11.08% more – a high amount compared to compost, where 1.89% ±0.03%.

K_2O in the bioslime is 5.30% ± 0.51% which is 14.19% more – a high amount compared to compost, where 4.67% ±0.46%.

CaO in the bioslime is 4.60% ± 0.12% which is 31.89% more – a high amount compared to compost, where 3.47% ±0.33%.

MgO in bioslime 1.43% ±0.05% which is 34.56% more – a high amount compared to compost, where 1.05% ± 0.11%.

Studies show that compost and compost derived from farm manure from extensive breeding of buffaloes for milk, meets the requirements of Regulation No 22 as a source of nutrients, but composting establish basic nutrient losses, because the system is open and is lost in the form of gaseous emissions. Establish a redistribution of some toxic elements, but increased their amount is not reasonably be excluded as a means to increase soil fertility.

Conclusions

1. It is determined that the manure from breeding of buffalo without litter is a suitable substrate for biogas production.

2. The bioslime has higher quantity of the water soluble fractions of biogenic macroelements, which is due to the partial mineralization of the substrate.

3. Methane fermentation allows higher preservation of the basic biogenic elements in comparison with composting.

Acknowledgment

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A New Cysticercus Pisiformis: No Tooth-Hook' Cysticercus Pisiformis

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Summary: During researching the ultrastructure of cysticercus pisiformis (CP) a no tooth-hook CP were discovered during examination. The rostellum of no tooth-hook CP was thicker and looked like layers pancakes with gear-liking structure. Viewed from the front of top, rostellum was flat, had a shallow hollow in center and radial arabesquitic structure around circum. There was a bigger round nodule in the center of rostellum. Four suckers were in contraction status, sucker entrance become smaller around contractive wrinkle walls. Suckers extremely protruded from surface and formed four nodule-like structures behind the rostellum. As mild movement of scolex, nodule in center was invagination in rostellum depth and formed three valve-like structures. Four suckers behind rostellum showed obviously longitudinal contraction. As stronger motion, tegument of ring-like rostellum was opening outside, and formed tire-like structure. Four suckers were in ringlike contraction, the entrance was getting smaller In a ward, while no tooth-hook CP is compared with tooth-hook CP, besides different structure of rostellum, the manner of contraction is different, too. This may be to benefit of adsorption of CP to host tissues.

Key words: cysticercus pisiformis; ultrastructure; cestode; no tooth-hook' cysticercus pisiformis

Introduction

It is well known [1 – 5] that human cestode has been divided into taenia solium and taenia saginata. The intermediate host of taenia solium who causes to cysticercus cellulosae is pig and of taenia saginata who lead to cysticercus bovis is bovine. The dogs' taenia pisiformis with tooth-hook is a parasite in the greater omentum and intestinal serous membrane and give rise to cysticercus pisiformis in rabbit who is its intermediate host. To this day, the taenia pisiformis without tooth-hook have not been reported in dogs.

To researcher the ultrastructure of cysticercus pisiformis the writers collected a lot of cysticercus pisiformis from greater omentum and intestinal serous membrane during inspection of slaughtering rabbits. In the examination of ultrastructure of scolex a no tooth-hook on the rostellum was found, which was named as no tooth-hook cysticercus pisiformis. For up to now the record about no tooth-hook cysticercus pisiformis is not found after looking up a lot of textbooks [6, 7] and literatures [8 – 10], it is reported as follows.

Material and methods

Collection of cysticercus pisiformis

The cysticercus pisiformis were gathered mainly from rabbit-kill abattoir. When the rabbit with cysticercus pisiformis was found in the inspection of paunch, the well-developed cyst located on the greater omentum and intestinal serous membrane was clipped immediately by sterilized surgical scissor, then put them in sterilized Petri dish, and took them to laboratory for standby.

Treatment of cysticercus pisiformis

In order to observe the ultrastructure of the csolex of cysticercus pisiformis in the relative rest states the cyst was cut by ophthalmic scissors. Cystic liquid were flowed out from the cyst. The scolex, neck and strobila were collected from the cystic wall carefully, put them into normal saline and slightly rinsed to remove the cystic liquid that was adherent on the surface of scolex. Finally, the scolex, neck and strobila after rinsing were put into 2.5% glutaraldehyde for the fixation.

Cultivation of cysticercus pisiformis

In order to observe the relative-motion states of the scolex of cyticercus pisiformis, according to previous methods and improving, it was that the well-developed

cysts were put into normal saline that contained 20% rabbit bile and preheated in 37℃ and cultured in 37℃ incubator. After culturing for 12 h, when the scolex, neck and strobila were evaginated from the cysts they were put carefully into normal saline solution by ophthalmic tweezers to fully rinse, and then fixed with 2.5% glutaraldehyde. For a few of cysticercus pisiformis that could not evaginated from the cyst autonomously, their cysts were cut by ophthalmic scissors to help the scolex, neck and strobila to evagination by artificial method.

Preparation of the scanning samples

The scolices fixed well with 2.5% glutaraldehyde were rinsed fully with PBS(pH 7.2) in twice for 5 min respectively, then, sucked the PBS liquid dry on the surface of scolex with absorbent paper. According to what to be observed, that part of the scolex would be fixed on sample stage with conductive adhesive under anatomic microscope. The samples were observed and recorded with Quanta type 200 scanning electron microscopy (manufactured by FEI Company of American).

Results

Structure in relative rest status

In relative rest status, the rostellum of no tooth-hook CP was thicker and looked like layers pancakes with gear-liking structure caused by contraction of tegument muscle in periphery. Viewed from the front of top, rostellum was flat, had a shallow deboss in center of the rostellum and radial arabesquitic structure around circum. There was a bigger round nodule in the center of rostellum(Fig. 1A). Four suckers were in contraction status, the entrance of suckers become smaller around contractive wrinkle walls. Suckers extremely protruded from surface of the scolex and formed four nodule-like structures behind the rostellum(Fig. 1B).

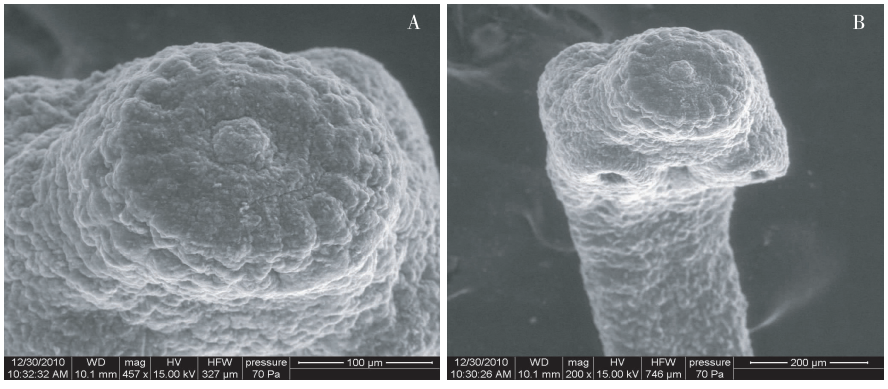

Fig. 1 No tooth-hook cysticercus pisiformis in relative rest status
A. There is a nodule in the center of rostellum with radial arabesquitic structure. ×457
B. Scolex and neck of no tooth-hook cysticercus pisiformis. Four suckers form nodule-like structure. ×200

Structure in relative motion status

The scolex evaginated through cultivation was in relative motion status. As mild movement of scolex, when the rostellum was in contraction, layers structure disappeared. Nodule in center was invagination in rostellum depth and formed three valve-like structures. The rostellum become ring-like structure, looked like a life buoy(Fig. 2A), in which surface was rough and had lots of radial crinkle walls. Four suckers behind rostellum showed obviously longitudinal contraction that made entrance of sucker toward above, protruded from surface and located in all round of rostellum. As stronger motion, tegument of ring-like rostellum was opening to the periphery, and formed wheel-like structure, in which there was lots of thick transverse striation, valve-like nodule were opened and showed like bellmouthing, there were a lot of thick-thin differing radial crinkle walls between dilating bellmouthing and tire-like structure(Fig. 2B). The rostellum looked like a wheel. Four suckers were in ringlike contraction, the entrance was getting smaller, there were layers contractive stripe around suckers and the internal wall of sucker showed longitudianl contractive stripe.

Discussion

Compared with the tooth-hook cysticercus pisiformis [11 – 13], besides the different structure of rostellum, the no tooth-hook cysticercus pisiformis had a differed style of contraction. The tooth-hook cysticercus pisiformis could fasten on the intestinal mucosa mainly by mean of it tooth hook on rostellum. For there was no tooth-hook on rostellum, the no tooth-hook cysticercus pisiformis had to change its structure to fix the intestinal mucosa firmly when it intruded the host. When the no tooth-hook cysticercus pisiformis was in relative rest state, its rostellum was skin rolling, no fissuring and for a whole

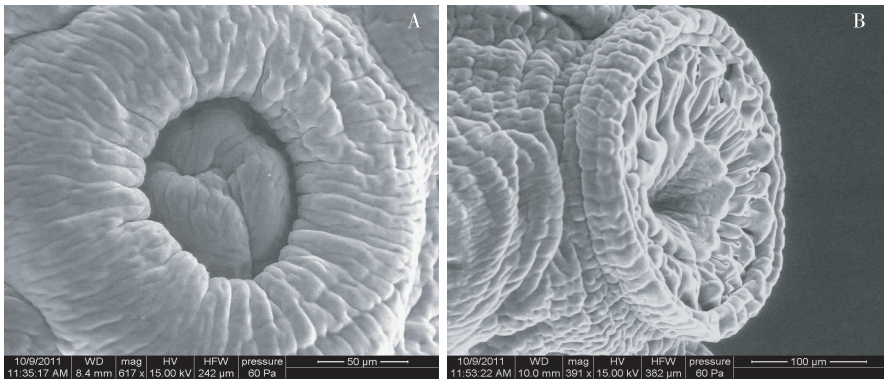

Fig. 2 No tooth-hook cysticercus pisiformis in relative motion status
A. Mild contractive rostellum looks like a life buoy. There are lots of radial crinkle walls on the surface of the rostellum. Nodule on the center of rostellum become three valves-like structure and invaginate into the rostellum depth. ×617
B. More intense contractive rostellum looks like a wheel, in which there is a lot of thick-thin differing radial crinkle walls between dilating bellmouthing and tire-like structure. ×391

viewed from the surface. As it was excited by some factors and began to motion, the rostellum of no tooth-hook cysticercus pisiformis took place the distinct changes. The nodule located in the center of rostellum got invagination, the tegument muscle on the rostellum began to contraction. The caverns were turned up on the rostellum. The rostellum looked like a big sucker witch was to benefit of the rostellum to fix on intestinal mucosa. When the no tooth-hook cysticercus pisiformis was in stronger notion, the tegument muscle of its rostellum got continuous contraction and extended in periphery, the valves-like nodule on rostellum opened and formed trumpet-like structure. Lots of radial rough wrinkles connected cutaneous muscle with trumpet mouth witch was able to greatly increase the osculatory proportion of rostellum to intestinal mucosa. This structure with large area folds and trumpet mouth clearly improved the adsorption capacity of rostellum to host's intestinal mucosa and contributed to the invasiveness of no tooth-hook cysticercus pisiformis to the host. In addition, through greater area touch with intestinal mucosa the rostellum of no tooth-hook cysticercus pisiformis could absorb more nutriment from the intestinal mucosa to supply the scolex for its kinetic demand.

The sucker's motion, looked-liking tooth-hook cysticercus pisiformis [14,15], also has two patterns that were ring-contraction and longitudinal-contraction. Viewed from structural changes, the sucker's motion of no tooth-hook cysticercus pisiformis was mainly ring-contraction. The ring-contraction of four suckers could make the scarskin of scolex tighten and make the orientation of four sucker's mouths in the step with rostellum's mouth. Therefore, the resultant of rostellum and suckers was easily formed to fix firmly on host's intestinal mucosa. The result of ring-contraction was that four suckers looked like ring layer nodules, protruded from the surface of the scolex and enclosed around the rostellum.

Conclusion

A large number research indicated that the taenia solium lived in human intestine could cause the cysticercus cellulosae in pig, and the taenia saginata was able to arise the cysticercus bovis in cattle. At present, it is too unclear that dogs' taenia pisiformis is also or is not divided into two kinds of cestodes. This research proved that the cysticercus pisiformis lived in rabbits' greater omentum and intestinal serosa membrane can be divided into two kinds that are tooth-hook cyticercus pisiformis and no tooth-hook cysticercus pisiformis by scanning electron microscope.

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Morphological Development Abomasums of Goats in the Postnatal Ontogenesis in First Months

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Summary: In goats, the growth and functional development of certain parts of the ruminant stomach continues during the first few months after birth. The aim of this research was to clarify morphofunctional changes in the kid's abomasum during the first postnatal month of life. After performing control slaughters of kids (on days 1, 17, 25 and 30) which were all kept in the same conditions, we carried out the morphometric analysis and weighing of various stomach compartments. Furthermore, the % ratio between size and weight of each were calculated. The gastrohromoscopical method was utilised to investigate the functional development of abomasums. In conclusion, as kids grow the weight relations between individual gastric parts change. One day old kids abomasums weight makes up 70% – 80% from gastric total weight where as in three weeks old 40% – 50%. The gastrohromoscopical method proved that the area where the abomasums pH is 3. 0 and less in newborn kids it is about 10% of the surface of the abomasum, however, with time it significantly increases so by the time they reach three weeks of age it will compose 80% of the mucosal surface of the abomasum.

Introduction

In goats, like in other ruminants, growth of the ruminant stomach parts and their functional development still continues during the first months after birth. Investigations have proved that different feeding factors and environmental conditions can influence the development of the ruminant stomach parts in kids [3, 10]. However, investigations should be continued in this field because not only the goat keeping and feeding management changes (feed components, way of feeding, frequency and duration) but also research methods that are [4,6,7,11,12,13].

Recently, investigations are carried out on issues whether the kids of different crossed breeds need a different amount of feed, vitamins and macro-elements during their intensive growing and developing period. Attention is paid not only to the growth and development stimulation by choosing the optimum amount of feed for each breed or crossed breed of goats but also to the amount and quality of the obtained products (meat and milk yield) [4, 7, 12]. The authors, investigating identification mini-boluses influence on the underdeveloped goat forestomach in the first month of life, consider that we lack modern studies on the growth and development of different stomach parts [1, 2]. Studying literature, we established that investigations on the functional activity changes of the abomasum during the first month of postnatal life are scarce.

That is why the aim of the present study was to find out if the feeding of milk replacer, which was intended for calves, changed the functional activity of the abomasum in goats as well as the weight ratio of the ruminant stomach parts during the first month of postnatal development.

Material and methods

Saanen breed 20 kids kept and fed identically on one farm were included into the research. The investigation took place from the animal birth up to the 30th day of postnatal life. All kids after birth lived with their mothers and first seven days were fed on colostrum (*ad libitum*) but on day eight they were weaned. After weaning, kids were placed into two pens (n = 10), and four times a day they were fed calf milk replacer (crude whey protein 22%, lysine 1.7% fat, 16% and 38% lactose) using nipple buckets. Drinking water and meadow hay were easy accessible.

During investigation, a control slaughter was carried out: at the age of one day (6 hours after birth; n = 5), 17 days of age (n = 5), 25 days of age (n = 5), and aged 30 days (n = 5). To find out the development of the multi-chambered stomach, its morphometric analysis and weight measurements were carried out [2,3,6]. After macroscopic examination, the ingest was removed from the stomach, and the following measurements were made: its total weight was measured, and then each part of the stomach separately was weighed. Using the obtained results the weight percentage of the abomasum and forestomach parts was calculated [2].

To evaluate the abomasum functional condition, gastrochromoscopic examination was carried out by using 0.3% of Congo red as an indicator. In the literature, two methods are mentioned to detect the parietal cell activity

of the abomasum: first, laboratory method, and second, express method [5,9]. We chose to use the express method. The abomasum was opened, cleaned and spread. Then, 0.3% of indicator Congo red was sprayed on the clean mucosa, in 1 – 2 minutes the result was evaluated. The indicator coming into contact with hydrochloric acid producing parietal cells changed its color from red to dark blue-violet [9]. The area of the active (hydrochloric acid producing) parietal cells was calculated as percentage ratio of the abomasum surface (with error ±3%).

Results and discussion

An analysis of adapting processes in the stomach in kids in postnatal ontogenesis was performed starting from 6 hours of birth until the animal reached 30 day old. The obtained results the weight percentages of the abomasum and forestomach parts are shown in Fig. 1.

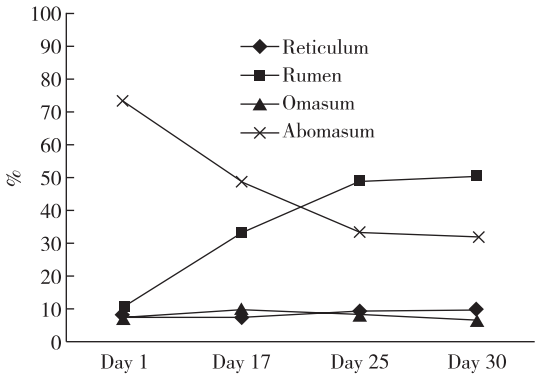

Fig. 1 The weight ratio of percentage in the various stomach compartments kids of one day old, 17 days, 25 days and 30 days old.

As kids are developing, the relation between weights of the gastric parts change - the rumen in seventeen days old kids is 33.5% and the abomasum 48.8% of the total gastric weight. After day 25 this relation changes in the favour of the rumen which then already reaches a 49.0% mark of the sum weight of the stomachs, the weight of abomasums then decreases to 33.4%. Similar dynamics are found by other authors [1,2,6,7,13], so we can say that feeding of milk replacer, which was intended for calves, not affect growth of stomach kids in the first month of life.

It turned out that newborn kids have 5% – 10% stomach area where the indicator Congo red (0.3% solution) changes colour from red to dark blue-purple. So, in the abomasum of newborn kid the 10% fundal gland cells are almost completely differentiated and the activity of the HCl producing parietal cells is pH 3 and less. Moreover, it is proved that at this time the level of calves and kids enzymes and hormones involved in the process of curdling and processing of colostrum could take place [6,8]. Obviously, the level of the hydrochloric acid secretion in the abomasum, that was determined in kids immediately after birth, is exactly as it is needed for digestion and absorption of the most important constituent parts of colostrum.

The animal grows the area significantly increases ($P < 0.01$) so by the time they reach 25 day of age it will compose 70% – 72%, bat 30 day of age it will compose 80% of the mucosal surface of the abomasum. This indicates that the fundal glands in the abomasal mucosa are already fully developed when the animal is 25 day - one month old and they produce hydrochloric acid which provides pH 3.0 and lower acidity level in abomasums. Such pH is necessary activates the pepsin action in the abomasum. Furthermore, we should think that during this period of time not only hydrochloric acid reaction increased in the stomach juice of kids but also the enzyme secretion (chymosin and pepsin) [8,10].

Conclusions

In conclusion, as kids grow the weight relations between individual gastric parts change. One day old kids abomasums weight makes up 70% – 80% from gastric total weight where as in 25 day old 33.4%. The gastrohromoscopical method proved that the area where the abomasums pH is 3.0 and less in newborn kids it is about 10% of the surface of the abomasum, however, with time it significantly increases so by the time they reach 25 – 30 day old it will compose 80% of the mucosal surface of the abomasum.

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Changes of Microbiological Spectrum in the Mouth Cavity and Duodenum of Dogs with Periodontitis

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Summary: Authors consider that animals suffer from the mouth cavity pathologies throughout their lifetime. A change of bacterial spectrum in the mouth cavity is considered as one of the factors causing periodontitis in dogs. It should be noted that we were interested in the question of whether the shift in bacterial spectrum of the oral cavity in dogs developing periodontitis, such changes were observed in the duodenum? Because the opinion that duodenal be virtually sterile. We can conclude that the development of periodontitis in dogs, oral cavity, the frequency of detection of gram-positive bacteria is growing faster than it is with gram-negative bacteria. By contrast, duodenum gram-positive and gram-negative bacteria in the presence of frequency remains relatively consistent across all stages of development of periodontitis. However, when comparing overall gram-positive and gram-negative bacteria in the detection of frequency changes in the mouth and duodenum dogs with different stages of development of periodontitis, it is concluded that this change in trend is broadly similar to the mouth and duodenum. In all stages of development periodontitis of the natural protective gram-negative bakteria overcomes *Eischerichia coli*, the gram-positive *Staphylococcus intermedius*.

Introduction

It is believed that animals suffer from the mouth cavity pathologies throughout their lives [4], and an important place of them takes periodontitis or periodontal disease [4]. It is proved that one of the periodontitis causing factors in dogs is changes of bacterial spectrum in the mouth cavity. In the mouth cavity, both gram-negative and gram-positive microflora gets on well' in balance; however, with periodontitis development this balance might be destroyed [2]. Literature sources mention that at the early stages of periodontitis gram-positive microflora predominates, but at more severe stages it is changed by gram-negative [3]. With advancing of periodontitis, the animal consumes more food improperly chewed; consequently a larger amount of bacteria(including pathogens) might reach the duodenum from the mouth cavity despite of the natural protective barriers [1]. In literature, no studies were found if and how changes of bacterial spectrum take place in the duodenum in dogs with advancing of periodontitis.

Objectives of the research: 1. Investigate microbial spectrum in the mouth cavity and duodenum and its changes in association with various stages of periodontitis development. 2. Find out what bacteria species are able to overcome the natural protective barriers of the digestive canal.

Material and methods

Basically, investigations were carried out at the Clinic of the Faculty of Veterinary Medicine of the Latvia University of Agriculture. Fifty dogs were selected for a profound examination of microbiological spectrum in the mouth cavity and duodenum. In order to define more precisely the stage of periodontitis, we used criteria of the development of periodontitis worked out by Rawlinson [5]. Depending on the obtained examination results of the mouth cavity, animals were conditionally divided into five groups dogs with the first, second, third and fourth stage of periodontitis, and dogs in which periodontitis was not found. To establish the bacteriological spectrum of the mouth cavity and duodenum in practically healthy dogs and in animals with periodontitis of different stages of development, samples were taken from the mouth cavity and duodenum by using sterile swabs. In each animal, samples were taken from the area of the gingival sulcus of the lower and upper jaw lateral surface. Duodenum samples were taken from himus and within 12 hours delivered to the Zemgale Regional Laboratory of the Food and Veterinary Service of the Republic of Latvia. Colonies of bacteria grown from the delivered samples were removed, and repeatedly plated on the blood agar culture medium, then differentiated by using API test.

Results and discussion

The obtained results show evidence that bacterial spectrum in the mouth cavity and duodenum is rather broad(Table 1 and 2).

Table 1 Spectrum of gram-positive bacteria in the mouth cavity and duodenum in dogs with and without periodontitis

Group of animals	Gram-positive bacteria	
	Mouth cavity	Duodenum
Without of periodontitis	*Staphylococcus intermedius* *Staphylococcus vitulus* *Corynebacterium bovis* *Corynebacterium kutscheri* *Enterococcus faecalis*	*Staphylococcus intermedius* *Staphylococcus vitulus* *Enterococcus durans* *Enterococcus casseliflavus/gallinarum* *Streptococcus porcinus* *Bacillus cereus*
With 1. stage of periodontitis	*Staphylococcus intermedius* *Staphylococcus aureus*	*Staphylococcus intermedius* *Staphylococcus aureus* *Staphylococcus epidermidis* *Staphylococcus cohnii* *Bacillus cereus*
With 2. stage of periodontitis	*Staphylococcus intermedius* *Staphylococcus saprophyticus* *Staphylococcus kloosii* *Staphylococcus haemolyticus* *Staphylococcus sciuri* *Enterococcus faecalis* *Bacillus licheniformis*	*Staphylococcus intermedius* *Staphylococcus saprophyticus* *Staphylococcus xylosus* *Staphylococcus hominis* *Staphylococcus aureus* *Streptococcus bovis* *Streptococcus porcinus*
With 3. stage of periodontitis	*Staphylococcus intermedius* *Staphylococcus saprophyticus* *Streptococcus porcinus*	*Staphylococcus intermedius* *Staphylococcus kloosii*
With 4. stage of periodontitis	*Staphylococcus intermedius* *Staphylococcus haemoliticus* *Micrococcus sedentarius*	*Staphylococcus intermedius* *Staphylococcus capitis* *Bacillus licheniformis* *Alloicoccus otitidis*

Results show that practically in all examined animal groups (except dogs with the third development stage of periodontitis), the number of gram-positive bacteria in the duodenum was larger than or equivalent to those bacteria species found in the mouth cavity. Consequently, gram-positive bacteria can overcome easier the natural protective barriers of the digestive canal. Possibly, they are able to stay in the duodenum for a longer time and move more slowly along the farther going digestive canal parts. The smallest number of gram-positive bacteria species in the mouth cavity was found in dogs with the first development stage of periodontitis two species, but in the duodenum in dogs with the third development stage also two species. The largest number of gram-positive bacteria species in the mouth cavity and duodenum was found in dogs with periodontitis development stage two seven bacteria species, respectively. The present research did not confirm an assumption that *Streptococcus* genus bacteria are basically diagnosed in the mouth cavity. In the examined animals, exactly *Staphylococcus* genus bacteria were found more often in both mouth cavity and duodenum. Moreover, as it is seen in Table 1, *Staphylococcus intermedius* in all examined dog groups was found simultaneously in the mouth cavity and duodenum. A conclusion can be drawn that from gram-positive bacteria *Staphylococcus intermedius* was the best capable to overcome the natural protective barriers in dogs with various development stages of periodontitis.

As regards gram-negative bacteria species, they were more in the mouth cavity than in duodenum in all examined animal groups (Table 2). So, it was more difficult for gram-negative bacteria to overcome the natural protective barriers than gram-positive bacteria. The largest number of gram-negative bacteria species, similar to gram-positive, was established in dogs with the second stage of periodontitis development. Possibly, it was due to the fact that in that stage of periodontitis development, in the mouth cavity inflammation and looseness in teeth could already be observed. Consequently, animals consumed their habitual food less chewed, and bacteria coming into contact with the bit got easier into the duodenum.

Table 2 Spectrum of gram-negative bacteria in the mouth cavity and duodenum in dogs with and without periodontitis

Group of animals	Gram-positive bacteria	
	Mouth cavity	Duodenum
Without of periodontitis	*Escherichia coli* *Acinetobacter lwoffi* *Enterobacter cloacae* *Klebsiella oxytoca*	*Escherichia coli* *Acinetobacter lwoffi*
With 1. stage of periodontitis	*Escherichia coli* *Pasteurella multocida* *Enterobacter sakazakii* *Brevundimonas vesicularis* *Serratia marcescens* *Klebsiella ornithinolytica*	*Escherichia coli* *Pasteurella multocida*
With 2. stage of periodontitis	*Escherichia coli* *Pasteurella multocida* *Klebsiella pneumoniae* *Klebsiella oxytoca* *Pseudomonas aeruginosa* *Acinetobacter lwoffi*	*Escherichia coli* *Pasteurella multocida* *Serratia fonticola* *Aeromonas hydrophila*
With 3. stage of periodontitis	*Escherichia coli* *Proteus mirabilis* *Pseudomonas aeruginosa* *Acinetobacter lwoffi*	*Escherichia coli* *Proteus mirabilis*
With 4. stage of periodontitis	*Escherichia coli* *Serratia liquefaciens* *Pseudomonas aeruginosa* *Aeromonas hydrophila*	*Escherichia coli*

At more advanced stages of periodontitis, such a broad bacterial spectrum was not observed. Those dogs most likely consumed more soft food; therefore it mixed better with digestive juices of the digestive tract, and the microflora present in the food was more successfully destroyed.

In all examined animals, from gram-negative bacteria *Escherichia coli* was that we found in the mouth cavity and duodenum at the same time. There are authors who consider exactly *Escherichia coli* as pathogenic or opportunistic bacteria especially when found in the upper part of the digestive canal.

It should be mentioned that practically in all examined dog groups(except dogs with the fourth stage of periodontitis), two gram-negative bacteria species managed to overcome the natural protective barrier. We can partially agree with those authors who consider that in dogs free of periodontitis or with early stage of periodontitis, gram-positive bacteria species predominate in the mouth cavity, but with progressive periodontitis they are changed by gram-negative species [3]. However, the present research shows that already at the first stage of periodontitis development in the mouth cavity of dogs, gram-negative bacteria started to dominate. In dogs with the third and fourth stage of periodontitis development, gram-positive and gram-negative bacteria were in a relative balance. As concerns the duodenum bacterial spectrum, we cannot agree to the opinion that it should be practically sterile [1]. The obtained results demonstrate that in all examined animals both gram-positive and gram-negative bacteria species were found in the duodenum.

Conclusions

1. In all dogs, irrespectively of the stage of periodontitis development, gram-positive and gram-negative bacteria can be found at the same time in the mouth cavity and duodenum.

2. Gram-positive bacteria spectrum in the duodenum in dogs is comparatively broader than gram-negative bacteria spectrum.

3. In dogs with the first and second stage of periodontitis development, bacterial spectrum in the mouth cavity and duodenum is relatively broader than in dogs with the third and fourth stage of periodontitis development.

4. From gram-positive bacteria, *Staphylococcus intermedius* overcome the natural protective barrier of all groups of animals, but from gram-negative bacteria – *Escherichia coli* bacteria.

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Preventive Application of Propolis and Herbs Extract in Rabbits Experimentally Infected with Enteropathogenic *E. coli* Strains

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Summary: The aim of the study was to determine an influence of ethanolic extract of propolis (EEP) and herbal extract in rabbits experimentally infected with *E. coli* on antioxidative status, biochemical blood parameters and health status.

An application of ethanolic extract of propolis (EEP) in rabbits infected with *E. coli* strains limited number of collapses and positively affected body weight gains. No *E. coli* presence in small intestine was noted in case of experimentally infected rabbits supplemented with EEP. Long-term administration of an extract of examined herbs may be related to an unprofitable influence on liver secretory functions. Positive influence of EEP on blood antioxidative status was noted.

Introduction

Diarrhea and collapses caused by enteropathogenic *E. coli* strains (EPEC) are the main reason of economic losses in rabbit broilers production. Losses caused by these strains ranges from 20% to 40%. Currently, main emphasize is put on treatment of disease cases with antibiotics application. Such action leads however to many negative consequences. Taking into account specificity of rabbits alimentary tract activity, antibiotic application gives very differentiated effects, causing in some cases even deterioration of clinical state and increase in collapses. Concurrently, such an activity contributes to formation and selection of bacterial strains resistant on antibiotics.

In a view of an increasing drug-resistance of microorganisms, decrease in an amount of antibacterial chemotherapeutics used would be undoubtedly a large benefit. The decision of European parliament establishing the second community program of activities in the field of health for the years 2008 – 2013, where microorganisms resistance on antibiotics and therapeutic limitations of infections are listed among priority threats of public health, proves the significance of this problem. Probiotics, prebiotics, herbs as well as propolis are an alternative for antibiotics [1 – 3].

The aim of the study was to determine an influence of ethanolic extract of propolis (EEP) and herbal extract in rabbits experimentally infected with *E. coli* on antioxidative status, biochemical blood parameters and health status.

Material and methods

The study was performed on rabbits (Hyplus line) starting from weaning. Clinically healthy rabbits were attributed to particular groups (n = 12): group Ⅰ - control; group Ⅱ - experimental (10% ethanolic extract of propolis was added to water in amount of 2 ml/l water); group Ⅲ - experimental (10% extract (20 ml/l water) from *Rumex cissus*, *Pontentilla anserina*, *Polygonum aviculare* herbs wt/wt 1∶1∶1). After 2 weeks of additives application the rabbits were infected *per os* with enteropathogenic *E. coli* strains isolated from farm rabbits with diarrhea (infectious dose 5×10^4). Blood analysis were performed at the day of beginning of the experiment and after 2 and 4 weeks. Hematological parameters, acid-base balance, biochemical parameters and antioxidative status (TAS and GPX) were examined in the blood. Body weight, fodder and water intake were registered during the experiment. Dissection and bacteriological examinations were conducted in collapsed rabbits, the samples were collected from small intestine and cecum.

The data was statistically processed with the computer program SAS, calculating mean values ($\bar{x}$) and standard deviations (SD). The significance of mean differences between the groups and within the groups between successive samplings was estimated with the Duncan test for each parameter studied.

Results and discussion

On commercial rabbit farms, mortality and culling

are of great relevance from the production and financial viewpoint. The highest risks of mortality and culling in does occurred during the first three kindlings, but remained stable thereafter. According to Rosell and de la Fuente [6] rabbits mortality resulting from an occurrence of alimentary tract diseases reaches 27%.

Rabbits from group Ⅱ receiving EEP obtained the highest body weight, while the lowest one was noted in the control group. Also the highest body weight gains were noted in group Ⅱ [Table 1]. Higher body weight gains were noted in another nutritional study conducted on rabbits [5], however in our study feed intake decreased clearly after infection.

After 2 weeks of herbal extract application, an increase in total protein content, AST activity in blood was observed, while an increase in total antioxidative ability was noted in the EEP group. The highest activity of glutathione peroxidase was observed in the group receiving propolis at the day of the end of experiment (results not presented in the tables). After 4 weeks of herbal extract application, an activity of AST, GGT and total bilirubin concentration was the highest in group Ⅲ when compared to other groups. AST activity was also elevated in the group supplemented with propolis (Table 2). Oliveira et al. [4], reported that crude propolis extract did not cause significant alterations in the serum aspartate aminotransferase activities.

Table 1 Growth performance in rabbit

Groups	Initial live weight (g)	Final live weight (g)	Daily weight gain (g)
Control	1860.00 ± 203.2	2478.18 ± 245.0[a]	25.76 ± 2.8
Propolis	1808.90 ± 187.3	2624.02 ± 198.7[b]	33.96 ± 3.9
Herbs	1822.33 ± 167.9	2565.13 ± 134.58	30.98 ± 4.7

[a,b]Statistically significant differences ($P \leq 0.05$) between groups.

Very abundant growth of enteropathogenic *E. coli* strains was noted in bacteriological cultures from small intestine and cecum in collapsed rabbits. In microbiological examinations performed after the end of the experiment in rabbits subjected to euthanasia, an abundant increase in *E. coli* was noted in cecum in all the groups, however it was differentiated in small intestine. Sparse *E. coli* was noted in small intestine of 3 rabbits in group I, no growth was observed in group Ⅱ, and sparse *E. coli* were detected in 2 rabbits in group Ⅲ. The ethanolic extract of Egyptian propolis, when administrated in combination with formalized inactivated *Pasteurella multocida* vaccine in rabbits' enhanced specific and nonspecific immune response [3].

Table 2 Selected biochemical blood indexes in serum blood of rabbits

Group	TP (g/l)	Albumin (g/l)	AST (U/l)	GGT (U/l)	Bilirubin (μmol/l)	Glucose (mmol/l)	LA (mmol/l)	Cholesterol (mmol/l)
				Start of experiment				
Control	49.68±4.87	37.20±3.56	25.81±4.76	9.65±1.65	2.69±0.87	7.51±1.67	16.86±3.23	2.23±0.78
Propolis	49.10±3.89	36.8±6.34	26.5±4.89	8.3±2.01	3.42±0.76	7.40±1.59	18.67±3.27	2.38±0.69
Herbs	47.80±6.45	36.8±9.68	26.2±7.11	4.1±0.67	1.54±0.59	7.21±1.45	12.97±2.67	2.09±0.67
				After 2 weeks				
Control	43.44±3.56	35.82±3.45	28.09±4.56[A]	9.47±6.78	3.03±0.78	7.92±2.34	12.77±2.56	1.44±0.87
Propolis	49.28±7.32	36.12±8.34	43.64±8.98[B]	10.96±3.44	3.93±0.49	5.32±1.87	12.39±2.43	1.73±0.67
Herbs	50.02±3.98	35.16±4.76	36.88±4.56[C]	7.88±3.65	3.84±0.76	6.29±1.57	11.62±3.23	1.52±0.59
				After 4 weeks				
Control	50.90±4.56	37.08±7.43	29.60±5.98[A]	6.75±1.89	2.75±0.21	6.89±1.58	14.76±3.67	2.49±0.68[a]
Propolis	53.04±6.76	35.45±5.76	47.09±7.01[B]	5.88±2.56	2.91±0.34	6.22±1.76	14.14±2.67	1.69±0.41[b]
Herbs	56.59±4.67	35.05±4.67	54.64±5.78[C]	7.75±1.43	3.19±0.34	6.00±1.98	14.71±2.78	2.84±0.43[a]

[A,B]differences statistically highly significant ($P \leq 0.01$) between groups in given sampling; [a,b]Statistically significant differences ($P \leq 0.05$) between groups in a given sampling.

Conclusions

An application of ethanolic extract of propolis (EEP) in rabbits infected with *E. coli* strains limited number of collapses and positively affected body weight gains. Long-term administration of an extract of examined herbs may be related to an unprofitable influence on liver secretory functions. Positive influence of EEP on blood antioxidative status was noted.

Acknowledgements

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Improvement of Murrah Buffalo Milk Curd Quality Made by Material Packaging

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Summary: Curd is kind of the probiotic fermentation food contained high level of nutrition. It is easy to be digested and be absorbed by body. This study investigated the effect of packaging which were without packaging, flexibel packaging and cup packaging as packaging stuff of curd on its organoleptic properties and its acceptability. This research is conducted in Silangit Village, Siborong-borong Sub District Tapanuli Utara District started in December 2011 until April 2012.

The research was used a compeletely random design with one factor that is fermentation time (7 days). The organoleptic properties and acceptability of curd were measured by Friedman test. LSD (Least Significant Difference) test was used to compare the significant difference. The research showed that the using of different packaging stuff of curd were affecting its organoleptic properties, such as color, aroma, taste, and compactness. However, there was a difference ini their texture properties. The sing of flexibel packaging showed the highest level of panelist acceptance, value of benefit cost ratio showed 1.82.

Introduction

Fulfillment of milk consumption on a national scale is still far from adequate or can be said to have uneven due to several factors, among others, national milk production is low and low-income people who are causing the inability to buy milk. This statement is also written in the thesis Siregar (2009) which states in megkonsumsi milk socialization among the people is still very low, whereas the consumption of milk is very good for health.

Buffalo milk is milk that is great for use as a raw material processed milk, because of its high fat content, especially the type of river buffalo (Warner, 1976).

According to the Department of Agriculture (1991) dairy products are well known in the area of North Tapanuli is buttermilk. But the curds have drawbacks that make it less desirable product by consumers, especially in people who are less accustomed to eating and children who have difficulty in swallowing. The process is very simple packaging also affects the quality, especially the appearance (appearance) and the nutritional value decreases with increasing storage time, for example after 2 – 7 days (tastes very sour and rancid with yellowish color) (Sughita, 1995). Buttermilk as a traditional North Tapanuli still processed and marketed simply this makes whey only known in a particular environment. In fact, if viewed from the nutritional value and benefits of probiotic food is curd should be developed processing and marketing.

Buttermilk is often sold in the center of the market town borong Siborong-quality packaging conditions (packaging) is very traditional and not quite as good as the traditional curd seller using only market for hand wash basin and cans that do not meet hygienic standards packaging. This statement is supported by Broto (2010) which states that the curd potential to be developed as a functional food source of probiotics that are beneficial to health as well as a source of animal protein. Malaysia and Japan have utilized the bacterial origin of buttermilk to make a fermented milk drink.

In Indonesia whey processing is still done traditionally, less attention to sanitation and hygiene so that quality assurance and food safety in doubt. Necessary to modify the packaging and product improvements curd by adding food additives to support the increase in value added, quality and food safety and correctional curd.

On the basis of the quality of packaging curds are still low in Siborong-borong and has not been much research in Indonesia on the packaging curd authors conducted a study to improve the quality of packaging with organoleptic test on people in sub-borong Siborong North Tapanuli. Moreover, I also want to know the financial feasibility of the packaging process Murrah buffalo curd of milk.

Material and methods

Place and time research

The experiment was conducted at the Laboratory of Superior Livestock Breeding Center Pig and Ox sub Desa Silangit Siborong-borong North Tapanuli, North Sumatra. This study will be carried out for 5 months.

Materials and devices research

Ingredients: Buffalo milk is used as an object of research. Panelists many as 20 people as a tester. Pineapple juice is to be taken as a coagulant (clotting) milk casein. Salt as much as 1% – 1.5% volume

buttermilk for buttermilk flavor giver.

Tool: Packaging of the cup and packaging materials from flexible materials. Tool flexible packaging presses. Mint plates as appropriate packaging maker curds form. Stove. Knife to cut pineapple. Milk can as a container of milk after milking. Grated to make pineapple juice. Spoon the pineapple juice makers and take whey from printing plates.

Research methods

The research method used was completely randomized design (CRD) with 3 (three) buttermilk packaging variations are:

1. Treatment A: Packaging curd without packing
2. Treatment B: Packaging curd with rigid packaging
3. Treatment C: Packaging curd with flexible packaging

Each treatment two replications to obtain 3 × 3 = 9 experimental units

Results and discussion

In this study conducted a preliminary study as a reference to carry out the study, namely to determine the storage time right and variations without packaging, flexible packaging and packaging cup in making curd. In the preliminary research conducted 3 (three) buttermilk packaging variations ie without packaging, flexible packaging and packaging cup of buttermilk each weight – 150 grams each wrapper and long storage for 7 days.

In this study Murrah buffalo milk curd studied were differences without packaging, flexible packaging and packaging cup that has similarities in the process of manufacture and the composition of the materials used but only different packaging alone is without packaging, flexible packaging and packaging cup. The ingredients to make this Murrah buffalo milk curd is Murrah buffalo milk, pineapple and salt.

Introduction based on the research results obtained Good for 7 days and the main research uses

7 days fermentation time, respectively – each per pack curds made weighed as much as 150 grams of curd wrapped with 3 packs ie without packaging, flexible packaging and packaging cup. Subsequently, respectively – each treatment in Organoleptic Test and Power Receive Murrah buffalo milk curd with no packaging, flexible packaging and packaging cup.

Consumer acceptability of a particular food product influenced by various factors, including environmental factors, socio-cultural, emotional condition and the effect of the product itself. Acceptance of it's own existence can be attributed to the level of customer satisfaction, so the better the acceptance of a product means that the higher the level satisfaction in consuming the product (Soediatama, 1993).

a. Color

Test results received power buttermilk color for each packaging indicates average – the average panelist liked the color of buttermilk (figures are rounded). Friedman test results for the color of the value of P = 0.000 indicates that the P value < 0.05 means that there is a difference without packaging, flexible packaging and packaging to the color cup Murrah buffalo milk curd.

b. Aroma

Test results received power aroma Murrah buffalo milk curd, for each – each treatment showed average – the average panelist rather like the smell of curd (figures are rounded). The results of the Friedman test for aroma P value = 0.074 indicates that the P value < 0.05 means that there is a difference without packaging, flexible packaging and packaging to the scent cup Murrah buffalo milk curd.

Test results received power panelists like flexible packaging with slightly fragrant aroma. Fishy aroma due to the nature of the typical dairy milk is easy to absorb odors around. Fishy aroma becomes reduced because fermented milk curdle.

c. Texture

Test results received power like flexible packaging with hard texture. The texture of the curd is caused by bacterial activity of Lactobacillus that connects between the casein milk during fermentation. The texture of the curd can be determined by looking at the success of fat that exist on the surface of the curd. If the fat pile up a lot, it shows that the texture of the curd has formed a compact period, and vice versa.

d. Taste

Acceptance of test results for each flavor curd – each treatment showed an average panelist rather liked the taste Murrah buffalo milk curd (figures are rounded). Friedman test results for a sense of the value of P = 0.061. This value indicates that P < 0.05 means that there is a difference without packaging, flexible packaging and packaging cup to taste Murrah buffalo milk curd.

e. Compactness

Test results received power curd compactness for each – each treatment shows the average panelist liked the compactness of Murrah buffalo milk curd (figures are rounded). Friedman test result for the compactness of P = 0.000. This value indicates that P < 0.05 means that there is a difference without packaging, flexible packaging and packaging cup against Murrah buffalo milk curd compactness.

Compactness is the nature of the compactness of the Murrah buffalo milk curd was observed with the sense of touch. Compactness properties can be seen from a compact least the Murrah buffalo milk curd. Usually

when people want to evaluate the compactness of a substance use fingers and is usually done by rubbing. The texture is compact in Murrah buffalo milk curd will make the product more palatable(Soewarno, 1985).

Benefit Cost Ratio (B/C ratio) Murrah buffalo milk curd

the curd with flexible packaging has a value of B/C ratio is the highest of 1. 89, which means the ratio between the revenue and the total cost of production of the Murrah buffalo milk curd. So, any costs incurred Rp. 1000, – will result in revenue of Rp. 1890. and the business is worth to be developed.

Conclusion

From the research that has been done can be concluded that the use of type of packaging that is without packaging, flexible packaging and packaging cup on Murrah buffalo milk curd affect the organoleptic properties such as color, aroma, taste, texture and compactness, but there is no noticeable difference in the texture properties. The use of flexible packaging in the Murrah buffalo milk curd panelists preferred than without packaging and packing cup. Value of B/C ratio is the highest curd with flexible packaging at 1. 89 and this effort should be developed. By Similarly Packaging Improvements Murrah buffalo milk curd from acceptable society.

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Validation of an Innovative Disinfection Equipment to Inactivate Bacterial and Viral Pathogens in Food Waste

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Summary: The results of the study showed that the injector is able to inactivate the tested microorganisms in food disposals with reduction rates of up to 4 log steps. However, these potential is fluctuating and strongly depends on the composition of the leftovers. Moreover, in accordance with the European commission legislation for the disinfection of animal by- products and derived products not intended for human consumption the number of *Enterococcus faecalis* and *Salmonella* Senftenberg has to be reduced by at least 5 log steps (142/2011).

Introduction

In Europe food waste has to be treated by a method which enables the inactivation of pathogens before it can be used as input material of composting and biogas plants, respectively. Usually the treatment is done by heating of the material for one hour at 70℃ (pasteurization). This is the standard method determined in the European legislation (1069/2009; 142/2011). If someone wants to modify this time/temperature profile or use a novel alternative method these modifications have to be validated and their efficacy proved against selective test bacteria, i. e. S. Senftenberg $_{W\,775}$. A method is considered as appropriate when the amount of these bacteria can be reduced by at least 5 log10 steps (1069/2009).

In this study a process validation (according to the German biowaste order) was performed for a novel flow injector apparatus using different test microorganisms and food and kitchen waste suspensions.

Material and methods

The injector is designed for a throughput of 5 m^3 per hour. Steam which is produced by an external steam generator is needed to provide the necessary temperature and is mixed to the liquid leftovers at the blast pipe (1% – 3% of the volume). The leftovers are pressed to the blast pipe by a continuously working flow. Due to its special shape a cavitation is built within the injector. This cavitation leads to a cell disintegration that can influence the survivability of different microorganisms. The time period in which the test organisms are exposed to the influences of the injector and the steam is limited to parts of one second.

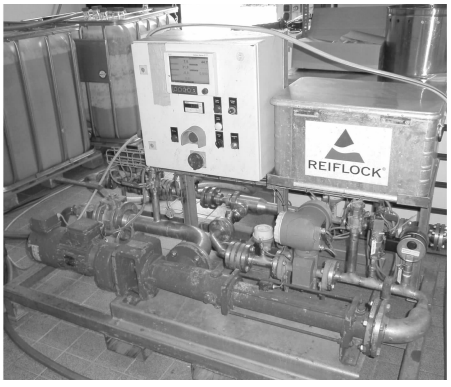

Fig. 1 Flow injector apparatus

During the experiments different physical parameters were monitored, i. e. temperature of the food waste at the entrance of the injector, temperature after the injector passage, and the pH values. Food waste samples were delivered from different canteen kitchens.

Experiments were performed twice using two different batches of food disposals. Both batches were different in homogeneity, texture, and fat content. *Enterococcus hirae*, *E. coli*, salmonellae, *Bacillus globigii spores*, and bovine enterovirus (ECBO) were used as test microorganisms. All test organisms were mixed into the food waste batches. In the first experiment the temperature at the flow injector was 65℃ and 75℃, respectively. In the second experiment only 75℃ was used. To detect the reduction rates achieved by the injector treatment samples were collected either from the input and the output material:

Samples A1 to A 3 and A1 to A6: temperature of substrate: 75℃

Samples B1 to B3: temperature of substrate: 65℃

Results and discussion

Figures show only the results of the experiments performed with *E. coli* and salmonellae. In the first experiment 10 L of *E. coli* suspension was mixed with 90 L of food and kitchen disposal and afterwards tested at 65℃ and 75℃ using the flow injector apparatus. By doing this an at least 6 step log-reduction of the bacterial load of the material could be achieved (Fig. 2, 3).

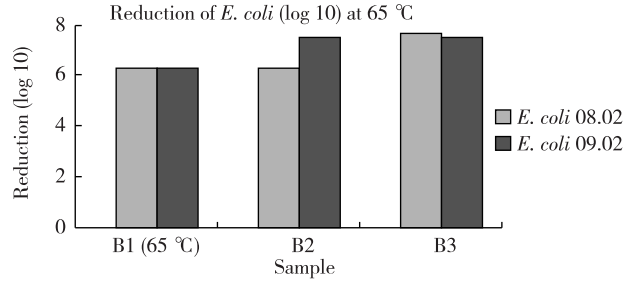

Fig. 2 Reduction of *E. coli* using the flow injector apparatus (65℃)

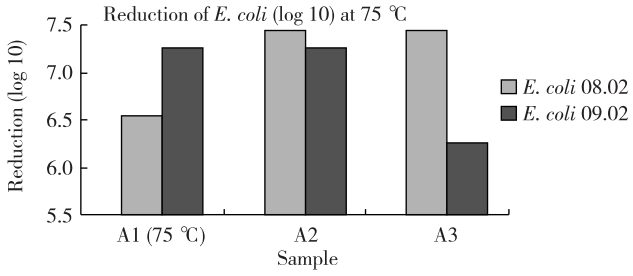

Fig. 3 Reduction of *E. coli* using the flow injector apparatus (75℃)

Against this, in a second experiment which was similarly performed the ratio of reduction was lower, i. e. approx. 4 log steps for salmonellae (Fig. 4). In this experiment a new lot of food and kitchen disposal was used which showed a higher ratio of fat. Therefore, it seems possible that the imbedding of the microorganisms in this fat has a protective effect on the bacteria; a phenomenon which has already been reported for fat and proteins in the eighties (Bitton et al., 1984).

Conclusions

The treatment of different test bacteria and ECBO virus in a novel flow injector apparatus revealed a microbiocidal effect on all these microorganisms. The injector is able to inactivate the tested microorganisms with reduction rates of up to 4 log steps. However, these potential is fluctuating and strongly depends on the composition of the leftovers (water, fat, protein content). This fact will be the object of ongoing experiments. All in all, the demands of the European commission legislation for the disinfection of animal by-products and derived products not intended for human consumption (142/2011) could not be achieved by using the flow injector.

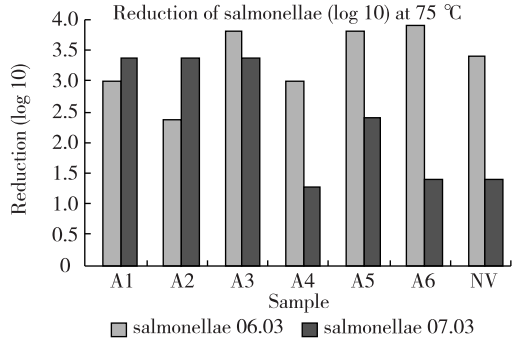

Fig. 4 Reduction of salmonellae using the flow injector apparatus (75℃)

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2. Commission Regulation (EU) No 142/2011 of 25 February 2011 implementing Regulation (EC) No 1069/2009 of the European Parliament and of the Council laying down health rules as regards animal by-products and derived products not intended for human consumption and implementing Council Directive 97/78/EC as regards certain samples and items exempt from veterinary checks at the border under that Directive.
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Effect of Phyto-mineral Feed Preparations on Blood Parameters of Laying Hens

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Summary: The aim of the present experiment was to assess the effect of the new feed additives, composed mainly of *Kalanchoe daigremontiana* and alfalfa, on blood parameters of laying hens, after investigation conducted for 5 weeks. Serum total protein, urea, uric acid, glucose, calcium and magnesium concentration was not changed by dietary treatments. However, hemoglobin and hematocrit concentration as well as glutathione peroxidase activity were the lowest in the control group. Serum total cholesterol level was lower in all experimental groups comparing with the control group but it was not confirmed statistically. The results of our study showed that feed additives composed of natural raw materials, containing biologically active substances, influenced selected blood parameters and hence improved health status of animals.

Introduction

Feed additives of natural origin from herbs, spices, plant extracts etc. may play important role especially in case of intense poultry production, where the birds are usually maintained in conditions of stress and lowered welfare [2, 5, 10]. Phytogenic feed additives are also an alternative feeding strategy to replace antibiotic growth promoters and have been incorporated in livestock and poultry feed to improve productivity [1, 11]. Moreover, natural raw materials may reduce an oxidative stress in animals and exhibit preventive and therapeutic activity [3, 6].

The aim of the present experiment was to assess the effect of the new feed additives, composed mainly of *Kalanchoe daigremontiana* and alfalfa, on blood parameters of laying hens.

Material and methods

The investigation was conducted in a room with controlled climate (light regimen of 16L:8D) where 150 laying hens (Hy-Line Brown, 25 week of age) were housed in a 3-tier battery system (5 hens per cage). A feeding experiment was conducted for 5 weeks. Feed and water were provided *ad libitum*. The basal diet was mainly composed of wheat (22.7% – 30.3%), soybean meal (22.7% – 24.5%), corn (20.0%) and barley (15.0%) according to nutrient recommendations for laying hens [9]. The phyto-mineral feed preparation were manufactured on the basis of natural raw materials (vermiculite, bentonite, calcium and humodetrynite form of brown coal) using four-stage technology including hydro-thermal processes [7]. The pulp of *Kalanchoe daigremontiana* leaves was preserved with refined glycerine (99.7% purity, Bio-Chem, Poland) in 1∶1 ratio.

Hens were assigned to the one of the 6 following treatment groups: control group (C) – standard feed mixture; Pulp group (Pulp) – standard feed mixture with addition of the pulp of *Kalanchoe d.* leaves; Phyto-mineral preparation 52 group (Pp52) – standard feed mixture with addition of the phyto-preparation containing 52.0% wt. of the pulp of *Kalanchoe d.* leaves; Phyto-mineral preparation 52α group (Pp52α) – standard feed mixture with addition of the phyto-mineral preparation containing 52.0% wt. of the pulp of *Kalanchoe d.* leaves and 6.0% wt. of dried *Medicago sativa* L.; Phyto-mineral preparation 36 group (Pp36) – standard feed mixture with addition of the phyto-preparation containing 36.0% wt. of the pulp of *Kalanchoe d.*; Phyto-mineral preparation 36α group (Pp36α) – standard feed mixture with addition of the phyto-mineral preparation containing 36.0% wt. of the pulp of *Kalanchoe d.* leaf's and 13.0% wt. of dried *Medicago sativa* L.. All investigated feed additives were supplemented at the doses of 5.0% wt.

At the end the investigation, 15 hens were randomly chosen from each group (3 hens/replicate) and the blood was taken from the vena brachialis. Approximately 5.0 ml of blood samples were collected into non-heparinised disposable test tubes for biochemical analysis. The blood serum was separated by centrifugation at 3000 × *g* for 10 min, and stored at –20℃ for determination of total cholesterol (enzymatic photometric test), HDL and LDL

cholesterol (colorimetric test), total protein (colorimetric test biuret reaction method), glucose (colorimetric test using Trinder's glucose oxidase method). Additionally, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were analyzed using the UV test according to the modified IFCC method without pyridoxal phosphate. The tests were conducted using commercial kits produced by Horiba ABX Company (Warsaw, Poland). The iron concentration was determined by photometric test using Ferene reagent manufactured by ALPHA DIAGNOSTICS Company (Warsaw, Poland). All analyses were conducted using biochemical analyzer Pentra 400, HORIBA ABX.

The experiment was conducted as a completely randomized design with 6 treatments, each treatment was 5 times replicated (5 hens per replicate). Data were tested for normality (Shapiro- Wilk's test). Statistical comparisons between the groups were done via one-way ANOVA using Statistica, version 10.0 (Statistica for Windows, StatSoft Inc., Tulsa. OK). All the data are reported as means, and effects were tested for significance using Tukey's multiple-range test, at a probability of $P < 0.05$ and $P < 0.01$.

Results and discussion

The effect of investigated feed additives on selected parameters in blood serum is shown in Table 1. Serum total protein, urea, uric acid, glucose, calcium and magnesium concentration was not changed by dietary treatments. However, hemoglobin and hematocrit concentration as well as glutathione peroxidase activity were the lowest in the control group. Serum total cholesterol level was lower in all experimental groups comparing with the control group but it was not confirmed statistically.

Table 1 Effect of investigated feed additives on selected parameters in blood serum (mean, n = 5)

Group parameter	C	Pulp	Pp52	Pp52α	Pp36	Pp36α	SEM	P-value
Hemoglobin (mmol/L)	8.138^{A}	9.077^{B}	8.677^{C}	8.450^{D}	8.480^{E}	8.610^{F}	0.0966	P < 0.0001
Hematocrit (L/L)	0.314^{A}	0.360^{B}	0.344^{C}	0.331^{D}	0.339^{E}	0.338^{F}	0.0490	P < 0.0001
GPX (U/L)	18090.6^{a}	22103.1^{b}	20506.6	22744.1^{b}	21011.1	22361.4^{b}	441.5	P < 0.0001
Total cholesterol (mmol/L)	3.224	2.909	2.967	2.820	3.071	3.231	0.1023	P < 0.0001
HDL cholesterol (mmol/L)	1.051	1.046	1.007	1.035	1.044	1.023	0.0209	P < 0.0001
LDL cholesterol (mmol/L)	0.963	0.831	0.841	0.802	0.853	0.970	0.0301	P < 0.0001
ALT (U/L)	10.247	15.407	7.200	9.600	14.193	13.587	0.9475	P < 0.0001
AST (U/L)	207.47^{a}	183.32	192.75	182.21	189.85	179.69^{b}	2.6770	P < 0.0001
ALP (U/L)	1025.43	1088.41	967.03	1053.37	888.93	587.86	73.20	P < 0.0001
Total protein (g/L)	46.12	45.56	49.41	45.06	46.49	48.93	0.6407	P < 0.0001
Glucose (mmol/L)	12.002	11.964	11.928	11.343	11.499	11.593	0.1472	P < 0.0001
Urea (mmol/L)	0.328	0.306	0.331	0.359	0.237	0.330	0.0157	P < 0.0001
Uric acid (μmol/L)	252.94	252.41	306.57	207.72	286.61	301.70	10.29	P < 0.0001
Ca (mmol/L)	6.491	6.213	7.025	6.410	6.653	7.001	0.1063	P < 0.0001
Mg (mmol/L)	1.389	1.395	1.429	1.412	1.313	1.437	0.0163	P < 0.000
P (mmol/L)	1.593	1.537^{a}	1.719	1.579	1.659	1.915^{b}	0.0421	P < 0.0001
Fe (μmol/L)	45.193^{A}	44.280^{A}	44.980^{A}	41.573	47.380^{A}	55.207^{B}	0.9431	P < 0.0001
Zn (μmol/L)	39.659	39.331	37.695	37.346	39.575	43.054	0.7697	P < 0.0001
Cu (μmol/L)	5.897^{A}	7.801	5.931^{A}	6.039^{A}	7.709	11.021^{B}	0.4722	P < 0.0001
TAS (mmol/L)	2.003	2.248^{acd}	2.107	1.949^{c}	2.244^{d}	2.238^{bcd}	0.0369	P < 0.0001

A-E values within the same row with different superscript letters differ ($P < 0.01$);
a-e values within the same row with different superscript letters differ ($P < 0.05$).

Malekizadeh et al. [8] were investigating the effects of using different levels of Ginger rhizome powder (GRP) and Turmeric rhizome powder (TRP) on production performance and some blood metabolites in laying hens. Results indicated that the inclusion of GRP and TRP into the diets decreased serum total cholesterol, AST and ALT significantly ($P < 0.05$). Bolukbasi et al. [4] were examined effects of dietary bergamot oil on performance, egg quality, blood metabolic profile and fatty acid composition of egg yolk in laying hens. Serum cholesterol concentration was reduced but AST, ALT, total protein, albumin and phosphorus concentration were not affected by supplementation of bergamot oil.

Conclusions

Summarizing, the results of our study showed that feed additives composed of natural raw materials, containing biologically active substances, influenced selected blood parameters and hence improved health status of animals.

Acknowledgements

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Implementation of Disinfection Standards in Bioga Plants—Impact for Practical Work

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Summary: Most biogas plants are operated in the mesophilic temperature range and usual input materials were liquid manure and renewable primary products sometimes biological waste. Using these temperatures the inactivation of pathogens included in the input materials is not sufficient leading to an epidemiological risk of the fermentation residues (transmission to soil, groundwater etc.). To minimize this risk biological waste has to be pasteurized (70℃/1 h) before it can be put into the fermentation process. One alternative to this procedure is the operation of the biogas plant in a thermophilic temperature range (> 50℃). Nevertheless, the efficacy of the inactivation potential of this process variant has to be proved. This is done by the validation of the inactivation capacity tested against the typical process validation bacterium *Salmonella* Senftenberg. The validation process performed in one biogas plant is reported.

Introduction

Due to the changed European legislation (1069/2009, 142/2011) the German biowaste order had to be adapted. Firstly, pasteurization is now included as a novel discrete disinfection method used in the anaerobic recovery in biogas plants. Secondly, thermophilic operated biogas plants in which the biowaste is not heated before fermentation have to be process validated. This process is described as follows.

Material and methods

In the German biowaste order the different methods leading to the disinfection of biowaste are listed:

1. Pasteurization
2. Aerobic treatment leading to disinfection (thermophilic composting)
3. Anaerobic treatment leading to disinfection (thermophilic fermentation)
4. Other methods of disinfection

Implementation of a process validation:

It is obligatory necessary to perform a process validation if you use thermophilic fermentation or alternative methods for the disinfection of biological waste material.

The first step of the validation process is to calculate the actual hydraulic residence time of the substrate within the fermenter by using a tracer. Thereby the input substrate is mixed with the tracer and the first-time appearance of this tracer at the spout is monitored. Spores from *Bacillus globigii* are one possible biological tracer and a chemical tracer is lithium. The spores cannot naturally be found in the biological substrates, are not pathogen in humans and animals, resistant against the temperatures used in the fermentation process, and can easily be detected in the laboratory. To detect the hydraulic residence time the biological material is spiked with *B. globigii* endospores (end concentration 10^6 spores per g fermenter content). Afterwards 1 kg samples are collected from the output material according to the following sample protocol:

1. every hour until 24 h
2. every 2 h until 36 h
3. every 4 h until 48 h
4. every 6 h until the end of the experiment.

By doing this, an actual hydraulic residence time of the substrate within the fermenter of 18 h was observed.

For the direct process validation of a thermophilic operated biogas plant test bodies (n = 8) containing the mandatory test bacterium *Salmonella* Senftenberg $_{W775}$ are applied to the fermenter vial. After 18 h (hydraulic residence time of the reactor) at 53.5 ℃ the bacterial test bodies were removed from the fermenter and screened for the survival of *Salmonella* Senftenberg $_{W775}$. The direct process validation is fulfilled if no salmonella can be recultivated from the test bodies.

Results and discussion

As shown in Table 1 no salmonellae could be re-isolated from the test bodies after 18 h at approx. 53℃.

In addition the treated biowaste were investigated for *E. coli* and enterococci after fermentation. The results are presented in Fig. 1, 2.

Table 1 Quantitative results of *Salmonella* Senftenberg in germ carriers after treatment in a thermophilic biogas plant (Process validation; >52℃/18 h)

	Salmonella quantitative * (cfu/g)
Suspension (S. Senftenberg $_{W775}$ (H_2S neg.))	1.71×10^9
Biowaste-untreated material	–
Biowaste-contaminated with salmonella	1.1×10^8
Germ carrier day 0 (control)	4.3×10^8
Germ carrier day 1 (control)	4.3×10^8
Germ carrier 1 – 8	–

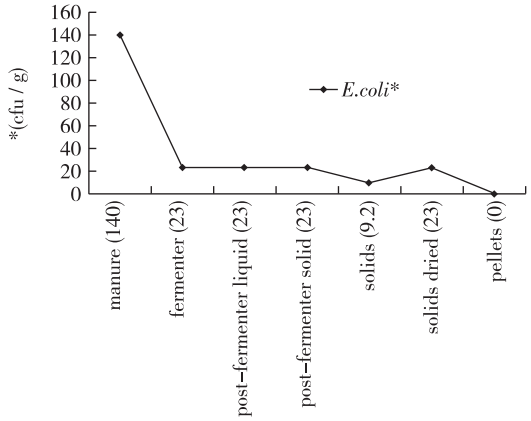

Fig. 1 Amount of *E. coli* in anaerobic treated biowastes and different substrates

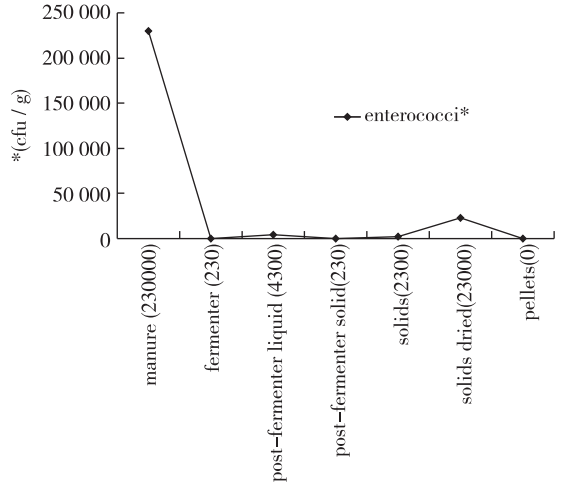

Fig. 2 Amount of *enterococci* in anaerobic treated biowastes and different substrates

E. coli and enterococci which were present in the raw input material were inactivated during the thermophilic fermentation process during 18 h and approx. 53℃ to the detection limit of both bacteria in culture (Fig. 1,2).

Conclusions

The process validation of the thermophilic operated biogas plant was successful. The test bacterium *Salmonella* Senftenberg which was applied to the fermenter using spiked test bodies could be reduced by eight log10 steps. *E. coli* and enterococci were only sporadically detected.

Therefore, fermentation residues produced in the validated plant have no epidemiological risk and can be used as fertilizers etc.

The greatest problem of the validation procedure described is the application of the test bodies into the fermenter.

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2. COMMISSION REGULATION (EU) No 142/2011 of 25 February 2011 implementing Regulation (EC) No 1069/2009 of the European Parliament and of the Council laying down health rules as regards animal by-products and derived products not intended for human consumption and implementing Council Directive 97/78/EC as regards certain samples and items exempt from veterinary checks at the border under that Directive.
3. Ordinance on the Utilisation of Biowastes on Land used for Agricultural, Silvicultural and Horticultural Purposes (Ordinance on Biowastes-BioAbfV) of 23rd April 2012 (Federal Law Gazette (BGBl. I S. 611).

Disinfection as an Important Preventive and Repressive Measure Against Infectious Diseases

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Summary: Chemical disinfectant means are characterised by various mechanisms of action on micro-organisms which result in different devitalisation effects on individual groups of micro-organisms. This aspect is frequently neglected in practice. The present study focused on evaluation of effects of chemical disinfectants on some bacterial species based on mechanisms of action and laboratory tests of bactericidal effectiveness. Despite knowledge of these mechanisms, the data presented in professional literature are frequently inadequate and even controversial which is discussed also in our studying. It can be stated that legislative provisions related to resolving of these issues are also deficient.

Introduction

Exploitation of chemical disinfectant means is one of very important preventive measures focused on devitalisation of micro-organisms. When looking at this issue from the historical point of view we can find literary data on the use of chemical disinfectants by veterinarians already in 1830 when Eberhardand Günter used chlorinated lime to suppress postparturient complications in animals, i. e. 37 years before presenting relevant scientific evidence by the pioneer of antisepsis Jozef Lister (Blancou, 1995). Importance of their use in surgical practice and treatment of the environment in relation to preventative of focal disinfection was somehow reduced by optimistic expectations related to introduction of antibiotics. Particularly focal disinfection, the principal measure ensuring eradication of outbreaks or epizootics, is essentially important for protection of public health.

Results and discussion

Our results presented in this section, but also results of other authors, are aimed at more complex understanding of this problem.

The aim of preventative disinfection is to decrease total counts of micro-organisms to ensure required hygiene standards on animal farms. The preventative disinfection is, more or less, concern of individual breeders but together with other sanitation measures it affects significantly the health and productivity of animals. The aim of focal disinfection is devitalization of the infectious agent in certain location to prevent its persistence in the heard or flock or development of disease outbreak or epizootics. Should an outbreak occur, there are legislative provisions that define duties of the involved parties in the duration of infection up to the final statement that the infectious disease is under control and eradicated which is possible only after final focal disinfection.

The action of disinfectant means dependent on their chemical composition is specific to various groups of micro-organisms. Within EU there are legislative provisions dealing with these aspect which are published in the form of EU Directives. In the Slovak Republic, measures have been developed in terms of these directives that deal with management of certain infectious diseases and are defined in government regulations. They define the duty of state veterinary care to ensure focal disinfection. Government Regulation of SR No. 116/2005 Coll. specifies measures for the control of foot and mouth disease; Government Regulation of SR No. 314/2003 Coll. deals with introduction of measures for suppression of Newcastle disease and Government Regulation of SR No. 367/2007 Coll. gives measures for the control of fowl plague. The § 29 of the last Regulation describes requirements on mechanical cleaning, disinfection and procedures of elimination and devitalization of fowl plague virus. Mechanical cleaning is an important measure that affects significantly the effectiveness of subsequent disinfection. It is discussed in detail in Supplement 5 of this Regulation. In item b) regarding disinfection it is stated that the disinfectants to be used and their concentrations are subject to approval of the Regional Veterinary and Food Administration so they ensure destroying the fowl plague virus. The item c) specifies that that the disinfectants should be used according to manufacturer's instructions, in agreement with instructions of the official veterinarian or the Regional Veterinary and Food Administration. In general, for

commercial reasons the manufacturer's instructions provide exaggerated data that do not correspond to reality. There is a wide collaboration basis within EU that can be used in this respect. When presenting the above Regulations we focused on viral diseases that allowed us to document that sodium hydroxide was the disinfectant of choice for such diseases.

In relation to this we believe that such materials should provide, for example in the form of recommendations, the disinfectant means that were tested and appeared effective on individual infectious agents on the basis of scientific studies and practical experience. A range of papers on this topic that were published in scientific and professional sources could serve as a basis for such recommendations (Denyer and Stewart 1998, Maris 1995).

Table 1 shows effectiveness of most important groups of disinfectants on G^- bacteria represented by *E. coli* and on G^+ bacteria represented by *S. aureus*. It shows that bactericidal effectiveness of NaOH on these two groups differs. When using the dilution test, *E. coli* was devitalized by 0.41% concentration and *S. aureus* by 1.25% concentration. The above mentioned allowed us to state that the well known virocidal properties of NaOH (Haas et al. 1995, Kahrs 1995) predestine this preparation for focal disinfection but do not comply with the requirements on preventative disinfection.

Table 1 Action of selected disinfectants on *S. aureus* and *E. coli*

Disinfectant	Devitalization effects	
	S. aureus	*E. coli*
NaOH	-	+
Persteril (active ingredient peracetic acid)	+	+
Chlorine compounds	+	+
Iodonals	+	+
Aldehydes	+	+
QAC	+	+

Chemical disinfectants are a diverse group of chemical compounds which is related to their mechanism of action (Grow 1995). Sodium hydroxide acts on surface membrane of *E. coli* where it causes saponification of lipids and triggers the subsequent enzymatic process of self-destruction of cellular wall. The outer membrane of G^- bacteria is composed of liposaccharides which may explain better devitalization effect on these bacteria compared to G^+ ones which lack this layer.

The knowledge of the mode of action allows one to select suitable disinfectants for focal disinfection targeted on devitalization of certain specific pathogen. Such knowledge forms the basis for development of new disinfectants, for example those containing synergistic additives that may improve their disinfectant effects.

Conclusions

Focal disinfection is an important measure in protection of public health. Its result depends on the disinfectant used. Our opinion is that the knowledge obtained in studies of the mechanisms of effect of disinfectants together with practical experience should be summarised and in the form of recommendations integrated in the existing legislative provisions and updated according to recently obtained knowledge.

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10. GOVERNMENT REGULATION OF SR No. 367/2007 Coll. specifying measures for the control of fowl plague. h) use of suitable disinfectants according to instructions.

Effects of Carryover of Subtherapeutic Antibiotic Dosages on the Bacterial Susceptibility in the Intestinal Flora of Poultry

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Summary: Due to the frequent use of antibacterials the risk of development of bacterial resistance increases worldwide. The aim of this study was to determine the influence of the carryover of fluoroquinolones on the development of bacterial resistance of *E. coli* in the intestinal flora of poultry. Different schedules of contamination of the environment of poultry with enrofloxacin were simulated to ascertain changes in the sensitivity of *E. coli*. Additionally, the environmental distribution of enrofloxacin and its metabolite ciprofloxacin was determined.

Minimal inhibitory concentrations (MICs) of *E. coli* were determined in four different treatment groups of enrofloxacin applied via drinking water (n = 10 chicken):

A) untreated control, B) recommended dosage (10 mg/kg, 5 days), C) 0.3 mg/kg (21 days, subtherapeutic dosage) followed by the recommended dosage and D) 1 mg/kg (21 days, subtherapeutic dosage) followed by the recommended dosage. Furthermore, animals were treated with the recommended dosage via drinking water as well as subtherapeutic dosages via food to detect environmental pollutions. Sedimented dust and air filters were analyzed for the two compounds.

Chicken treated with the recommended dosage over 5 days developed a slight MIC-shift, which is arranged over the epidemiological cut-off (0.125 μg/mL) but under the clinical breakpoint (> 2 μg/mL). The MICs of the animals treated with 0.3 mg/kg followed by the recommended dosage conducted similarly. Animals treated with 1 mg/kg developed a MIC-shift (bacterial resistance) within 21 days. Under the recommended dosage of 10 mg/kg resistant bacteria were preselected. Both compounds could be detected in dust in concentrations correlating with the applied dosage.

In conclusion, during animal treatment antibiotic contaminations occur in the direct environment. Animals exposed to antibacterials in subtherapeutic concentrations pose a risk for the development of bacterial resistances in dependence of dosage.

Introduction

Non pathogenic *E. coli* spp. are part of the intestinal flora of animals and humans and can pose as a gene reservoirs for the horizontal gene transfer of antibacterial resistance genes [1]. The horizontal transfer seems to be the primary cause of multidrug resistant gram negative bacteria [2]. Thus, the aim of this study was to determine the influence of the carryover of enrofloxacin on the development of bacterial resistance of *E. coli* in the intestinal flora of poultry. Therefore a treatment schedule was used, which contained the comparison between the recommended treatment dosage and this recommended treatment dosage after the animals have been exposed to different subtherapeutic concentrations to simulate a carryover.

Additionally, the environmental distribution of enrofloxacin and its active metabolite ciprofloxacin, which is used as a broad spectrum antbiotic in human medicine, were measured [3].

Material and methods

Four different treatment groups (n = 10 chicken) were provided for the treatment schedule of enrofloxacin applied via drinking water. The first one A) acted as untreated control, the second one B) received the recommended dosage (10 mg/kg) over 5 days, the third C) and the fourth D) group were treated with subtherapeutic dosages C) 0.3 mg/kg; D) 1.0 mg/kg for 21 days followed by the recommended dosage via 5 days (Fig. 1).

To determine the minimal inhibitory concentrations (MICs) of *E. coli* in the intestinal flora of the animals, cloacal swabs were taken at different time points before, during and after the treatment duration. The MICs of the isolated colonies of *E. coli* were determined by using the Epsilometer test (Etest®, bioM rieux SA, Marcy-l'Etoile, France). Additionally, after the cloacal swabs have been directly smeared, the same swabs were taken and enriched to streak it onto an Endo agar plate containing 2 μg/mL enrofloxacin.

Furthermore, animals were treated with the recommended dosage via drinking water as well as subtherapeutic dosages via food for 5 days to detect environmental contaminations.

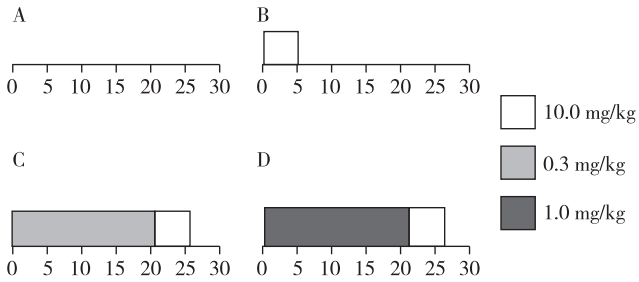

Fig. 1 Experimental design of the treatment schedule: A) untreated control, B) recommended dosage, C) subtherapeutic dosage followed by the recommended one, D) another subtherapeutic dosage followed by the recommended one.

Therefore sedimented dust, collected at different locations of the stable, as well as air filters installed in the stable were analysed by high-performance liquid chromatography and fluorescence detection at 264 nm.

Results and discussion

The animals, which were part of the untreated control, did not show any changes. Chicken treated with the recommended dosage over 5 days developed a slight MIC-shift, which is arranged over the epidemiological cut-off(0.125 μg/mL) but under the clinical breakpoint (> 2 μg/mL). The MICs of the animals treated with 0.3 mg/kg conducted similarly, single colonies showed an MIC of 2.0 μg/mL but after enrichment of the particular cloacal swab and the following streak onto an Endo agar, containing 2.0 μg/mL enrofloxacin, no grown colonies could be determined after incubation. After the successional treatment with the recommended dosage, one single colony showed an MIC of 3.0 μg/mL over the clinical breakpoint, but could also not be confirmed by enriching the cloacal swab. Animals treated with 1 mg/kg developed an MIC-shift (bacterial resistance) within 21 days. Under the following recommended dosage of 10 mg/kg resistant bacteria were preselected. After the end of treatment with the recommended dosage the number of resistant bacteria of *E. coli* seemed to be reduced, but even after a waiting period of 13 weeks resistant colonies could be detected.

It has been shown before that quinolone resistance in *E. coli* isolated from broilers previously dosed with flumequine and enrofloxacin, both fluoroquinolones of the second generation, was significantly higher than the resistance of *E. coli* isolated from poultry without an exposure [4]. This study showed a similar result. All animals were free of antimicrobial resistance against enrofloxacin before treatment started. Each group treated with enrofloxacin, independent of dosage, showed MIC-shifts. But just group D showed resistance after the animals received the subtherapeutic dosage of 1 mg/kg, which was preselected by the following recommended dosage. 1 mg/kg represent 10% of the recommended dosage, the official carry-over level is 2.5% [5], which is in accordance with the amount of enrofloxacin given to group C. But apart from one colony with an MIC 3.0 μg/mL, which could not be confirmed by enriching the swab, no resistance could be detected. That shows that the effect of carryover can prepare or cause the development of bacterial resistance, but it depends on the absorbed dosage. And in dependence of the instant study the dosage has to be higher than the carry-over level of 2.5%.

But even if bacterial resistance did not appear in the other groups, MIC-shifts could be determined.

Enrofloxacin and its metabolite ciprofloxacin could be detected in dust in concentrations correlating with the applied dosage under the experimental conditions.

Conclusion

Antibiotic animal treatment leads to a contamination of the direct environment. The exposure to subtherapeutic concentrations of enrofloxacin pose a risk for the development of antibacterial resistance of apathogen *E. coli* in dependence of dosage. That also leads to the question, whether the proportion of antibiotic amounts leading to the development of bacterial resistance, is reached in industrial livestock farming and how it can be reduced.

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UV-Irradiation as Potential Tool to Reduce Environmental Pollutions of Sulfonamides Arising from Swine Livestock

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Summary: The present study aims to determine ways to avoid environmental pollution by sulfonamides as test antibiotics, since the frequent use of antimicrobials in animal livestock poses a risk for environmental pollutions. The effect of UV-light on the degradation of sulfonamides was studied, as well as the antimicrobial activity and biocompatibility of the irradiation products.

Nine sulfonamides were exposed to UV-light (UVA; UVA/UVB) and sunlight to study photo-oxidation. The brilliant-black reduction test with the germ *Geobacillus stearothermophilus* var. *calidolactis C*953 was used to study the antimicrobial activity of the degradation products. Furthermore, the impact of the UVA/UVB-irradiation products on cell viability and proliferation of two murine cell lines (fibroblasts and keratinocytes) was investigated.

UVA/UVB treatment of several sulfonamides results in degradation of the compounds in different amounts with the highest degradation for sulfathiazole and sulfanilamide. The UVA/UVB light degradation products exhibit no antimicrobial activity. Sun light exposure reveals a similar degradation pattern of the different sulfonamides. UVA-irradiated sulfonamides degrade to a lesser extent (except sulfamethazine). The degrading products exhibited no toxic effects except sulfanilamide in keratinocytes, and altered cell proliferation to a different extent.

UVA/UVB light can inactivate sulfonamides in aqueous solutions. Degrading products exhibit no antimicrobial activity anymore and have no impact on cell viability and a low impact on cell proliferation *in vitro*. Therefore, firstly the treatment of aquacultures with sulfonamides has to be reconsidered, although UV-light effects may be restricted to superficial layers of the water and secondly UV-light may be a useful tool to reduce the environmental pollution e. g. via manure from pig farms. However, the biocompatibility results have to be used for a detailed toxicological risk assessment and the effect of irradiation product on soil organisms has to be investigated in further studies.

Introduction

Beside tetracyclines and β-lactam antibiotics, sulfonamides are the most frequently used antibacterial agents in veterinary medicine in Europe. Most of the sulfonamides are only partially metabolised in the organism after therapeutic treatment and, therefore, pose a risk as environmental pollutants due to the excretion via urine or faeces. Remaining antimicrobial activity in some of the metabolised products consequently might also entail a risk of the development of antibacterial resistance. Contamination from animal husbandry primarily affects soil and water, pig, poultry and cattle manure, slurry and liquid dung, hospital waste water and sewage being the main sources[1 – 3].

The main problem of antibiotic residues in the environment is the development of bacterial resistance due to concentrations beyond the required concentration to assert a definable effect in the microorganisms. A portion of sulfonamides is reversibly sequestered in a residual fraction in soil for a long period and might potentially become released again in low amounts[4, 5].

Antibiotic residues in soil can be incorporated by plants after distribution of manure onto the acreage of various agricultural crops, as already shown for tetracyclines and sulfadiazine [6, 7]. Thus, the uptake of contaminated plants with antibiotics in subtherapeutic concentrations may be another health risk for animals and humans.

One possibility to eliminate sulfonamides from the excretion of animal husbandry is a photodegradation reaction due to the absorption of radiation [8 – 10]. Several studies have demonstrated that sulfonamides undergo photocatalytic degradation with various compounds generated during this process. Since the antimicrobial activity depends on the chemical structure, photodegradation may develop other antimicrobial substances or may inactivate the drug.

The present study was designed to investigate the effect of natural irradiation and two types of artificial light (UVA and UVA/UVB) on the fate of several sulfonamides in order to study cytotoxic and

antiproliferative effects of photodegradation products as well as antimicrobial activity.

Material and methods

Photolysis experimental setup

Photolysis experiments were carried out in three different ways: a) UVA/UVB-exposure, b) UVA-expose and c) natural sun light exposure. The following sulfonamides (Sigma-Aldrich, Steinheim, Germany) were used: sulfanilamide, sulfathiazole, sulfamethoxypyridazine, sulfachloropy-ridazine, sulfamethazine, sulfamethoxazole, sulfadiazine, sulfamerazine, sulfadimethoxine.

The sulfonamide solutions were exposed to UVA light or UVA/UVB-light in a glass petri dish up to 6 hours (surface area 44 cm^2, solution depth approximately 0.5 cm). Natural conditions were used to gain information on photolysis under sun light exposure. These experiments took place in June-July, with temperatures between 20 and 28℃. The weather was unsettled with sunny and cloudy times. The solutions were exposed to the light for seven days with sampling withdrawals every 24 hours. High performance liquid chromatography (HPLC), under various isocratic conditions, was used to determine the amount of sulfonamides before and after irradiation.

Cell culture experiments

Toxicity test: The CellTiter 96 AQueous One Solution Cell Proliferation Assay™ (MTS-assay, Promega, Mannheim, Germany) was used to study the impact of the degradation solutions on cell viability of two cell lines (keratinocyte MSC-P5 and fibroblasts L929). Therefore, cells were treated with the solutions for 24 and 48 hours: 100 μg sulfonamide solutions not exposed to UV-light and 6 hour UV-light exposure samples. Medium treated cell served as control.

Proliferation assay: To detect the impact of the sulfonamide photodegradation products on cell proliferation, a cristalviolet proliferation assay was used. Therefore, cells were treated with the solutions for 24 and 48 hours: 100 μg sulfonamide solutions not exposed to UV-light and 6 hour UV-light exposure samples. Medium treated cell served as control.

Antimicrobial activity

The antimicrobial activity of the test substances and degradation products were measured using the commercial available assay "BRT MRL Suchtest ESL" (AiM GmbH, München, Germany). The assay contains the bacillus "*Geobacillus stearothermophilus* var. *calidolactis C*953" and brilliant black to indicate the growth or inhibition of the thermophilic bacterium.

The determination of the antibacterial activity of the degradation products was performed in comparison to the unirradiated sulfonamides.

Results and discussion

The present study demonstrated that all investigated sulfonamides undergo photodegradation during UV-light irradiation. For all sulfonamides except sulfamethazine the degradation process was most dynamic under UVA/UVB-light irradiation followed by UVA-light irradiation to natural sun light. For sulfamethazine the degradation was similar under UVA/UVB- and UVA-light exposure and less dynamic under natural day light. UVA/UVB-light exposure of aqueous sulfonamide solutions resulted in photolysis in different extent. Within four hours, sulfathiazole and sulfanilamide have almost been completely degraded, while sulfamethoxypyridazine and sulfachloropyridazine were degraded up to 70% after six hours. The lowest degradation was observed for sulfadiazine, sulfamethoxazole, sulfamethazine, sulfadimethoxine and sulfamerazine, all of which were reduced to amounts of 20% – 30% of the initial concentration. Depending on the chemical structure (e. g. five-membered vs. six-membered heterocyclic substitutes), sulfonamides show different photodegradation behaviour [11, 12]. With respect to the results of the present study, no structure-photodegradation relationship was observed. Solely used UVA-irradiation on aqueous sulfonamide solutions resulted in reduced degradation rates of the sulfonamides in comparison to UVA/UVB-light (except for sulfamethazine). Sulfachloropyridazine, sulfamethoxypyridazine and sulfanilamide were degraded to the highest extent (31% – 65%), while all other sulfonamides were degraded to a much lesser extend (1% – 18%).

In comparison to irradiation with UV-light by an artificial light source, sun light exposure over seven days resulted in reduced degradation rates, which in turn are comparable to UVA/UVB-irradiation concerning the order of degraded sulfonamides. Although the degradation rates were lower in comparison to UVA/UVB-light exposure, colouring of UVA-irradiated solutions could be observed to a greater extent: sulfachloropyridazine, sulfamethazine and sulfamethoxazole (yellow after two days), sulfamethoxypyridazine and sulfadiazine (yellow after three days), sulfanilamide (wine red after five days) and sulfathiazole (light brown/yellow after seven days). Reduced degradation rates of sulfonamides exposed to natural sunlight could likely derive from a lower light intensity in comparison to the artificial light sources.

Besides photodegradability of sulfonamides, toxicity assessment of the arising products is important for estimation of the suitability of UV-light to decrease environmental contaminations of sulfonamides. Concerning the viability, the investigated murine cell lines (keratinocytes and fibroblasts) exhibited a high biocompatibility of the investigated sulfonamides for 48 hours (viability of approximately 80% – 100%), while the 6 hours irradiated solu-

tions caused no reduction in cell viability of both cell lines except sulfanilamide 48 h incubation in murine keratinocytes (to a level of 60%).

The effect of several sulfonamide photodegradation products on the growth of C. vulgaris has been investigated by Baran et al. (2006) [13]. All investigated sulfonamides (sulfamethoxazole, sulfacetamide, sulfadiazine and sulfathiazole) were toxic to *C. vulgaris*, albeit in different extent, and degradation products generated after various illumination times had inhibitory or stimulatory effects to the growth of *C. vulgaris*. Incubation of murine fibroblasts and keratinocytes with several sulfonamides showed a reduction of the proliferation after 24/48 hours incubation.

However, it should be noted that the concentrations of irradiated sulfonamides that were non-toxic to keratinocytes or fibroblastes were higher than the levels of sulfonamides detected in manure or in the environment. Nevertheless the potential ecological risk should not be underestimated.

Another important element of the investigation of the impact of UV-light on sulfonamides is the potential antibacterial activity of the degradation products. Therefore, the brilliant black reduction test with *Geobacillus stearothermophilus* var. *calidolactis C*953 was used and demonstrated that the photodegradation products exhibited no antibacterial activity. In the case of sulfamethxoxpyridazine, sulfachloropyridazine and sulfadimethoxine this effect is presumably caused by the SO_2 extrusion. Thus, there is problably no threat to the development of antibacterial resistances in the environment after UV-light irradiation.

Conclusions

All investigated sulfonamides undergo photodegradation under UV-light exposure to a greater or lesser extent and result in biocompatible degradation products with no antimicrobial effect.

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Automatic Dairy Cattle Welfare Monitoring—The Current Situation

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Traditionally the welfare and health of dairy cows has been monitored through observation by stockpersons and veterinarians. Cattle welfare can be assessed using different parameters and a combination of methods. For the assessment of welfare, and to accommodate the information requirements of consumers, different systems have been developed [1]. The Bristol Welfare Assurance Programme and Welfare Quality® Project for assessment of the welfare status of animals use mainly animal-based measures, which give more appropriate results of an existing welfare situation. Environment-based parameters are used for the identification of possible improvements to animal welfare.

The increased number of cows per herd, and automation of the technological processes, has significantly decreased contact between humans and cows in large cowsheds. These trends increase the need for effective automatic animal health and welfare monitoring systems. Today, emerging technologies offer possibilities to develop fully automatic on-line monitoring and control of animals' health and well-being [2]. The monitoring of data concerning individual animals and their surrounding environment on farms (building attributes and microclimate, feeds and feeding, farming technology, diseases etc.) and the results of processing such data can be used for the improvement of animal health and welfare in general, and to reduce the risks of illnesses, pain and stress. In this article the principles and possibilities of automated monitoring of dairy cow health and welfare in large loose housing cowsheds are considered.

The health and welfare statuses of dairy cattle are influenced directly by housing. Knowing the cow's reactions (changes in milk yield and composition, physiological and behavioural reactions), it becomes possible to estimate health and welfare status and changes in comparison to the norm. To devise a system for functional automatic welfare evaluation it should meet five main conditions: 1) cows should be automatically identified; 2) monitoring and registering of housing environment parameters should be monitored and registered; 3) cow behaviour, physiological parameters, production level and other animal-based parameters reflecting welfare status should be monitored; 4) software for processing and interpreting data, and models for welfare estimation, should be incorporated; 5) data on the housing environment, animal-based parameters and diseases, as well as welfare score, should be stored in a database and updated in real time.

Automatic decision making within the system to improve welfare is mostly possible concerning the microclimate. In other cases the participation of a human actor in the decision-making process is obligatory, especially in diagnosis and medical treatment. A list of automatically collectable animal-based welfare parameters is given in Table 1.

Three main groups of data should be considered in acquisition sub-systems which interface with each other. These are: 1) data stored in the cowshed management information system (MIS); 2) data from housing environment monitoring sub-systems, and 3) data from additional modules for physiological and behavioural monitoring (Fig. 1).

Existing management information system (MIS) for dairy cattle housing incorporate automatic identification of animals and means for registering housing conditions, individual productivity, reproduction, etc., and databases for keeping records. Usually also some cow-specific physiological parameters are recorded by MIS, for example body temperature, milk conductivity, animal activity.

For monitoring the housing environment the air temperature, air humidity, air velocity, and lighting can be measured and data stored in a database. For registration of the concentrations of carbon dioxide, ammonia, and other noxious gases, digital controller-based specific systems are also available [3]. Additional data acquisition systems for cows' physiological and behavioural parameters are ideal to include as autonomous modules with a universal interface for data exchange. Examples of these are various sub-systems for lameness estimation.

A lot of useful data about cows is collected and stored in databases, especially for breeding purposes and health control. For example, the Estonian Animal Recording Centre collects data on productivity, milk composition and somatic cells count, reproduction and disease incidence monthly. All these measures can be used successfully for the automatic evaluation of health and welfare.

Table 1 Automatically collected animal-based parameters in loose housing

No.	Measurable and calculable parameters	Measurement place	Application domain	Remarks
1	Milk temperature	MP, MR	health, oestrus	conventional
2	Milk conductivity	MP, MR	udder health, breeding	conventional
3	Milk colour	MP, MR	udder health	novel
4	Milk homogeneity	MP, MR	udder health	novel
5	Milk composition: lactose, protein, fat progesterone lactate dehydrogenase urea beta hydroxyl butyrate	MP, MR	 udder health, nutrition reproduction udder health nutrition ketosis, metabolic disorders	novel, pilot
6	Somatic cell count	MP, MR	udder health	novel
7	Milk yield, flow, milking time	MP, MR	health, nutrition	conventional
8	Milking frequency	MR	behaviour, health	novel
9	Milking order	MP	behaviour, health	pilot
10	Body temperature	MP, MR, AS	health, oestrus	conventional
11	Heart rate	AS	health	novel, pilot
12	Respiration rate	MR, CF	health	pilot
13	Intake	MP, MR, CF, FA	health, nutrition	novel, pilot, conventional
14	Rumination	AS	health, nutrition	novel, pilot
15	Bodyweight	MR, CF, WA	health, nutrition	conventional
16	Body condition score	WA	health, nutrition	novel, pilot
17	Animal activity	AS, WA	behaviour, health, oestrus	novel, pilot, conventional

MP—milking parlour; MR—milking robot; AS—sensors attached to the animal; CF—concentrate feeder; FA—feeding area; WA—walking area.

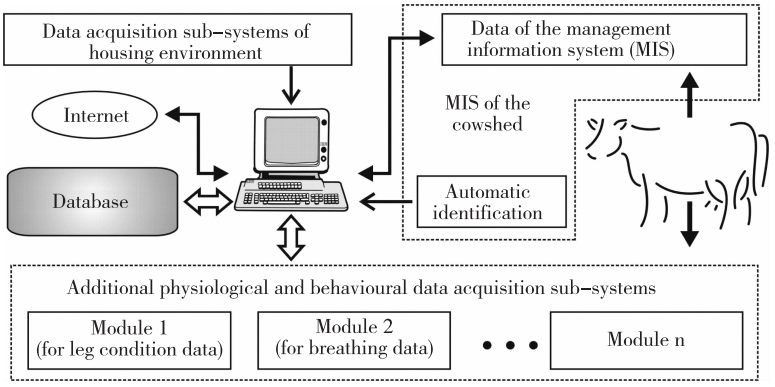

Fig. 1 Principal structure of an automatic system for monitoring of cow's health and welfare

An important factor in automatic health and welfare monitoring is the arrangement of data exchange between the different parts of the integrated system. This is influenced by: 1) the structure of the network, 2) unification of the interfaces and protocols, 3) management information system(MIS) openness for data exchange.

To enable the connectivity of existing and new measurement and analysis equipment within the system the most important element is the data exchange network. The amount of information that should be exchanged within this network will increase with the addition of new elements, and the information exchange speed may become of great importance, especially in the case of video data analysis.

The results of the analyses may be used for the prediction of situations needing attention, and for the issuance of warnings to the farmer. These tasks will be carried out using software models. Algorithms of these models, based on various mathematical methods and scientific investigations, are used to estimate the influence of

different factors and the interactions of these with welfare status. The number of these factors is usually large and the interactions varied.

An example of the possible configuration of local measurement for the assessment of leg condition, and the information exchange system, is shown in Fig. 2.

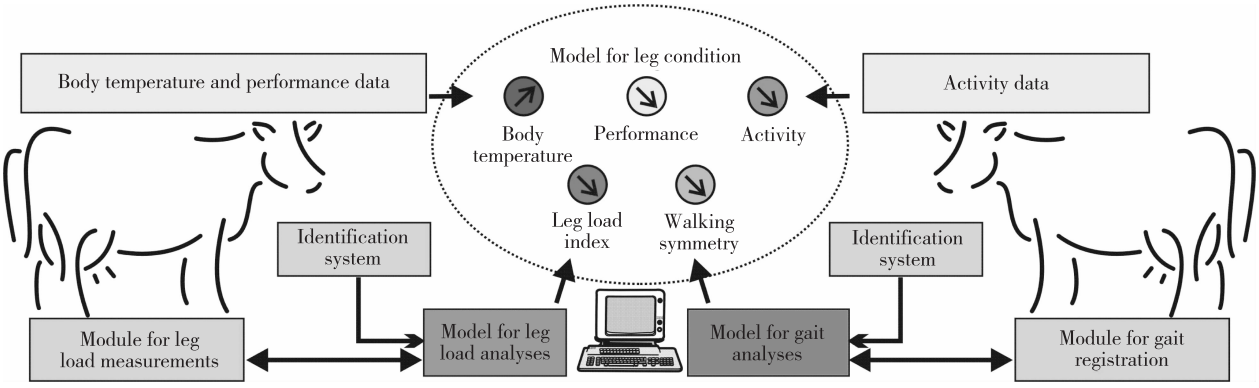

Fig. 2 Concept of automatic evaluation of leg condition and proposed inputs to the model

For instance, the indicators of leg problems include: reduced locomotion activity, reduction in milk yield, increased body temperature, decreased load on an injured leg, change in gait pattern. All these indicators can be used as model inputs. Specific software and models to analyse cows' movement and gait are currently being developed by several investigation groups [4, 5].

A general welfare model construction should be based on certain welfare evaluation schema. For instance, the Welfare Quality® system recently proposed utilizes over 30 on-farm measures which give value judgements for 12 different welfare criteria. These criteria form the basis for the description of four independent welfare dimensions to estimate the overall welfare status. Approximately the same structure should be applied for welfare modelling. This would lead to a set of different models arranged into a hierarchical structure (Fig. 3).

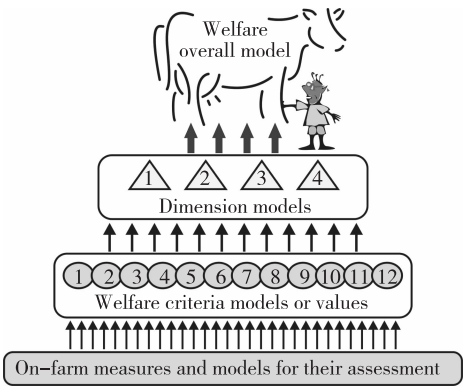

Fig. 3 Concept of an automatic cow's health and welfare evaluation system

Conclusions

Automatic health and welfare assessment of dairy cows enables the efficient control of welfare on milk production units, to reduce losses caused by diseases, to optimize management procedures and productivity of cows.

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A Test-Bench for Infrared Photoacoustic Analyzers Used to Measure Gas Emissions from Animal Houses and Manure Storage

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Summary: Animal production is recognized as a major source of ammonia and greenhouse gases despite the high uncertainty associates to current estimates. Mitigation strategies are proposed to decrease the emissions. International standards exist for the national emission inventories or for the composition analysis of manure or soils but not for the measuring methods and associated uncertainty of the emissions of animal production. This limits considerably the quality control and the certification of existing reductions. One of the key measurements is the concentration increase of ammonia or greenhouse gas due to the animal production. Many research teams in the world have chosen Infrared photoacoustic spectroscopy analyzers(PAS; e. g. INNOVA® 1312 or 1412) to measure selectively the concentrations of ammonia and greenhouse gases. Network activity between them is difficult because different PAS can give different concentrations because of differences in configuration or maintenance: the chosen filters can compensate for different cross interferences, different concentrations used for calibration can induce differences in measurements, a significant drift in calibration can appear due to the infrared source or the microphones or changes following the use in highly contaminated environments. Therefore, INRA and IFIP, with the financial support of ADEME, developed a test bench that can be used to compare different PAS, to check the calibration drift during a project, to evaluate the effect of not compensated interferences when using a PAS in a new animal production system. Equipment and software allow the rapid preparation of gas mixtures with ammonia, nitrous oxide, methane, carbon dioxide at different concentration levels diluted in dinitrogen. The uncertainty of the concentrations in the mixture is evaluated based on the uncertainties of the flow meters and the gas bottles. The concentrations measured by the PAS are compared in real time with the concentrations of the gas mixtures.

Introduction

World Regions with intensive animal production have to focus on environmental impacts of livestock because of social, political, health stakes. The environmental impacts of intensive livestock production concern all the environment compartments (air, water and soil). For instance, animal production is recognized as a major source of ammonia and greenhouse gases and therefore significantly contributes to the global warming and acidification. Nevertheless national inventories are not accurate because of the difficulties associated to the assessments of emission factors. One of the key of emissions measurements is the concentration increase of ammonia (NH_3) or greenhouse gases (CH_4, N_2O) due to the animal production. Many research teams in the world have chosen infrared photoacoustic spectroscopy analyzers (PAS; e. g. INNOVA® 1312 or 1412) to measure selectively the concentrations of ammonia and greenhouse gases[1 – 4]. Network activity between them is difficult because different PAS can give different concentrations because of differences in configuration or maintenance: the chosen filters can compensate for different cross interferences, different concentrations used for calibration can induce differences in measurements, a significant drift in calibration can appear due to the infrared source or the microphones or changes following the use in highly contaminated environments. Therefore, INRA and IFIP, with the financial support of ADEME, developed a test bench that can be used to compare different PAS analysers(INNOVA®).

Material and methods

The aim of the developed test bed is to generate gas mixtures with chosen NH_3, CH_4, N_2O and CO_2 concentrations. 4 bottles of pure gases at diluted concentrations(400 ppm NH_3, 3000 ppm for CH_4, 100 ppm N_2O and 50000 ppm for CO_2) are connected to mass flow controllers (Bronkhorst® F-201CV-500-RAD-11-V with numerical control; Fig. 1). The initial concentrations of the bottles have been assessed considering the concentration ranges in the final gas mixture. The concentrations is the N_2O, CH_4 and CO_2 bottles are certified. Their small volume allows a storage in the room at 20℃.

N_2 is used for dilution and to provide the total required output flow for the analyser (minimum 1.8 L/min). A Nafion Tube, connected to the N_2 line, is used to humidify the gas mixture. In order to avoid high pressure at the inlet of the analyser, at the exhaust of the test bed one tube is connected to the inlet of the analyser and another one is opened to the outside. Two flow meters were added between the two output lines (PTFE tubes) of the test bed in order to control the output flow (at 3 L/min when the analyzer doesn't pump).

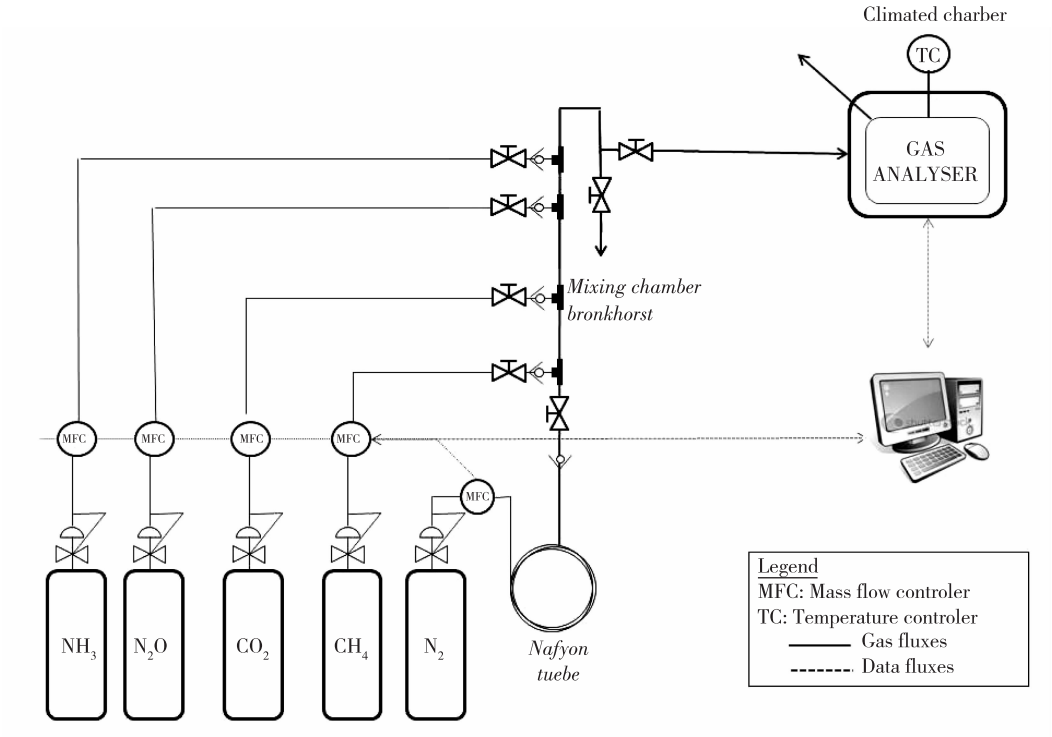

Fig. 1 The test bed is composed of mass flow controllers and gas bottles (CH_4, CO_2, NH_3 and N_2O) at diluted concentrations. N_2 is used as vector and dilution gas.

The mass flow controllers are controlled with a Labview program (Fig. 2) that allows choosing and measuring the mass flows corresponding to various concentration levels in the gas mixture. A FlowView software sold by Bronkhorst® is also used to check the communication with the computer. Two analysers can sample simultaneously the mixture.

The program is also used to visualize and record the concentrations levels in the gas mixture, the concentrations measured by the analysers, the calibration data, the concentration uncertainties (gas mixture, analyzer). The raw signal (mV) from the microphones is also collected as it can be used to develop specific calibration equations, different from those incorporated in the analyser.

Results

The possible concentrations levels that can be obtained in the gas mixture are presented in Table 1. The lower concentrations levels varied in function of the nitrogen flow. The minimum concentration levels can be reached with a high nitrogen flow (12 L/min). With the minimum nitrogen flow, the maximum concentration levels can be reached.

For each gas the uncertainty on the concentration in the mixture is evaluated based on uncertainty on mass flow controller and in the gas bottle.

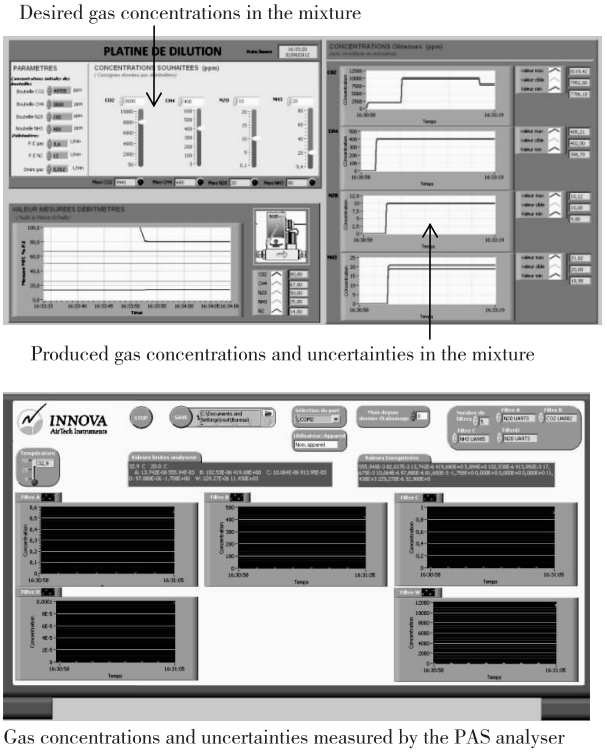

Fig. 2 Man-machine interfaces used to control the mass flow controllers and recorded data from the mass flow controllers, the gas concentrations in the mixture, the gas concentrations and the raw signals given by the PAS analyser

Table 1 Concentration ranges in the gas mixture as a function of the total output flow

Gas	Output flow >3L/min	Output flow at 3L/min	
	Concentration Min	Concentration Min	Concentration Max
CO_2 (ppm)	50	200	10000
CH_4 (ppm)	3	12	600
N_2O (ppm)	0.1	0.4	20
NH_3 (ppm)	0.4	1.6	80

Discussion

This test bed developed for PAS analyser can be used for many purposes: to compare different measuring devices, to test calibration in different ranges, to detect failures and calibration drift, to evaluate interference (of non-targeted gases) impacts on measured concentrations, to estimate uncertainty on gas concentration measurements. When using PAS analysers on farms, other uncertainty sources must be added to the uncertainty evaluated in laboratory conditions. They can highly contribute to the uncertainty on gas concentrations measurements (e. g. condensation in sampling pipes, spatial heterogeneity).

Conclusion

The development of quality control for gas concentrations measurements relies on the development of tools as the test bed presented in this paper. It can improve the comparison of emission measurements performed within a network of organisms over years. To propose a complete uncertainty estimate and to perform the raw data treatment (by performing the correction of all interferences), the presented test bed should be completed with a system that allows the control of the gas moisture and the production of gases from liquid solutions.

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Ammonia Concentrations within and Emission Rates from Australian Piggery Buildings

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Summary: Both animal and human health can be compromised by high ammonia concentrations within piggery buildings that can result in high emission rates from livestock buildings as well. Concentrations and emissions of aerial ammonia within and from 160 piggery buildings were surveyed in four states of Australia. Multi-Gas monitoring system was used in the buildings to determine ammonia concentrations. A refereed methodology was used to predict the emission rates from all buildings studied. An overall mean ammonia emission rate of 1442.5 mg/h/500 kg live weight and a mean internal building concentration of 3.7 ppm were measured in the piggery buildings. The lowest ammonia concentrations were measured in straw based shelters(1.1 ppm), while measurements taken in grower buildings had the highest concentrations(5.5 ppm).

Key words: air quality, ammonia, survey, risk factors, emission

Introduction

Airborne pollutant emission, negative human and animal health effects are associated with aerial ammonia that can be found in the airspace of intensive piggery buildings (Banhazi et al., 2008b; Banhazi et al., 2009). High ammonia concentrations are a concern for livestock managers as a number of studies demonstrated the association between ammonia, viable airborne particles and different lung-related diseases in animals and humans(Donham et al., 1989; Crook et al., 1991; Lee et al., 2005; Murphy et al., 2012) as well as the significant effects of sub-optimal air quality on production efficiency (Urbain et al., 1999). Therefore, the two main objectives of this study were to(1) document internal concentrations of ammonia in different types of piggery buildings used in commercial production systems in Australia; and to(2) calculate, using refereed methodology, the emission levels of ammonia from different types of piggery buildings in Australia.

Material and methods

In total 160 piggery buildings were included in the study. Each herd received 4 two-day visits during a period of 1 month and on each farm, dry sow, weaner, grower/finisher sheds, farrowing rooms and straw based shelters were surveyed during the study (Banhazi et al., 2008b; Banhazi et al., 2008c). Multi Gas Monitoring (MGM) machine was used for ammonia and carbon dioxide measurements as described previously (Banhazi et al., 2008b; Banhazi et al., 2008c). The estimate of emission rate was determined from the product of the ventilation rate, which was based on the carbon dioxide balance method(Seedorf et al., 1998). Window based STATISTICA 6.0(StatSoft Inc., 1996) was used to conduct basic statistical manipulation of the data, such as grouping and descriptive statistics. A detailed model was later developed to test various interactions and the results of the detailed analysis have been published previously (Banhazi et al., 2008a; Banhazi et al., 2008c; Banhazi et al., 2008d). However, in this paper grouping(one-way ANOVA/grouping) was used to report on average values recorded in different buildings.

Results

The results of internal concentrations of ammonia measured in different types of piggery buildings included in the study are shown in Table 1. Grower buildings had the highest ammonia concentrations recorded(5.5 ppm), while the lowest ammonia concentrations were measured inside straw based shelters(1.1 ppm). The highest maximum concentrations of ammonia was also measured in grower buildings, indicating that this type of buildings had consistently higher ammonia concentrations, compared to other types of buildings(Banhazi et al., 2008c; Banhazi et al., 2010). The mean ammonia concentrations were the same in finisher(3.1 ppm) and dry sow (3.1 ppm) buildings.

The mean ammonia emission was 1442.5 mg/h/LSU (Table 2). Finisher, straw-based shelters and weaner buildings all had relatively low emission rates (mean emission rates of 1123.2, 1146.7 and 1143.5 mg/h/LSU, respectively) while grower buildings had the highest mean emission rates calculated(2050.4 mg/h/LSU). Maximum values for ammonia emission rates were also compared(Table 2). In all buildings maximum emission rates were quite similar ranging between 9240.3 and 14006.4 mg/h/LSU. However, maximum ammonia

emission rate was quite markedly lower from straw based shelters (5880.2 mg/h/LSU) than from other buildings.

Table 1 Ammonia concentrations (ppm) inside the study buildings (Summary table of means)

Building type	Mean	No of buildings	Minimum	Maximum
Grower	5.5	34	0.11	29.36
Finisher	3.1	21	0.15	20.10
Straw based shelters	1.1	9	0.84	2.00
Dry sow	3.1	21	0.06	22.71
Farrowing	4.1	27	0.08	20.00
Weaner	2.8	29	0.00	17.46
All groups	3.7	141	0.00	29.36

Table 2 Ammonia emission values per livestock units (LSU = 500 kg live weight) and per animal from different piggery buildings (mg/h)

Building type	Mean	No of buildings	Minimum	Maximum
Grower (LSU)	2050.4	26	38.1	13618.1
Finisher (LSU)	1123.2	15	50.6	10434.5
Straw based shelters (LSU)	1146.7	7	91.7	5880.2
Dry sow (LSU)	1424.2	13	16.6	13356.3
Farrowing (LSU)	1307.3	18	15.5	14006.4
Weaner (LSU)	1143.5	21	1.5	9240.3
All groups (LSU)	1442.5	100	1.5	14006.4
Grower (per animal)	193.1	26	2.5	1498.0
Finisher (per animal)	147.7	15	8.4	1252.1
Straw based shelters (per animal)	193.2	7	3.7	1117.2
Dry sow (per animal)	492.0	13	5.8	4674.7
Farrowing (per animal)	614.1	18	7.2	6583.0
Weaner (per animal)	31.7	21	0.0	240.2
All groups (per animal)	267.0	100	0.0	6583.0

Emission rates per animal are also presented in Table 2 and when considering emission per animals; dry sow (492.0 mg/h/animal) and farrowing buildings (614.1 mg/h/animal) are the highest emitters, reflecting on the relatively small number of animals kept in these buildings per unit space. Weaner buildings are considered to be low emitters (31.7 mg/h/animal), as the overall emission rates are subdivided by a typically large number of animals kept in weaner buildings, resulting in a low emission rate per weaner pigs.

Discussion

The concentration of ammonia was the lowest in straw based shelters. The current "safe" concentration recommendation in Australia for exposure of ammonia is 10 ppm in livestock buildings (Banhazi et al., 2008b). The concentration of ammonia is generally not a great concern in Australian piggery buildings, since ammonia concentrations on average did not exceed recommended levels. However, the recorded maximum levels did provide reasons for concern. Some of the maximum levels approached 30 ppm in grower buildings and these maximum levels were measured over a 60 h period. Thus exposure for such high concentrations of ammonia over a period of one or two days can cause damage to the respiratory track especially when it is combined with high airborne bacteria levels (Murphy et al., 2012). The relatively low level of ammonia could be related to the fact that in Australia, the design of piggery buildings is generally open due to high temperatures throughout the year. Such open buildings provide high ventilation rates that eliminate high ammonia concentrations generally. From the results of the research presented, it can be concluded that the average ammonia concentrations measured in various piggery buildings in Australia is acceptable in most of the buildings.

However, the potential effects of high ammonia emissions from piggery buildings on the rural environment need to be also considered. The per animal emissions were very high for farrowing and dry sow sheds, because in these sheds are relatively small number of animals are housed, compared to weaner, grower or finisher buildings. Therefore when the overall emission is divided the relatively small number of animals the resulting figure is relatively large. On the other hand, in weaner buildings the emission per animal is always relatively small, due to the large number of weaner pigs in these buildings. However, it does not necessarily mean that the overall emission is in any way smaller indeed when livestock units are compared, it can be seen that weaner sheds actually do

contribute at similar levels as finisher buildings to the overall emission rates.

Conclusions

An overall mean ammonia emission rate of 1442.5 mg/h/500 kg live weight and a mean internal building concentration of 3.7 ppm were measured in 160 piggery buildings. Straw based shelters showed the lowest concentrations of ammonia with average values of 1.1 ppm, while the highest concentrations of ammonia (5.5 ppm) were recorded in grower buildings. The highest emission rates of ammonia (per LSU) were observed also in grower buildings (2050.4 mg/h/LSU). Given the concentrations, it is unlikely that the concentrations of ammonia in isolation are affecting the health of stock or personnel working in piggery buildings in Australia. However, the emission rates calculated from the study sheds were relatively high, due to high ventilation rates recoded in the study buildings (Banhazi et al., 2008d).

Acknowledgments

This study was part of a larger project funded by the Australian Pork Limited. It was also a collaborative effort between the South Australian Research and Development Institute (SARDI), Agriculture Western Australia, The Queensland based Pig Unit and Agriculture Victoria and involved the contribution of many people. We wish to particularly acknowledge the contribution of pig producers involved in the study, Dr Colin Cargill for his professional advice and the assistance of all technicians involved in the study.

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Respirable and Inhalable Dust Concentrations within and Emission Rates from Australian Piggery Buildings

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Summary: Concentrations and emissions of airborne inhalable and respirable dust within and from 160 piggery buildings were surveyed in four states of Australia. Respirable and inhalable dust samples were collected on filter papers using standard gravimetric measurement methods. A refereed methodology was used to predict the emission rates from all buildings studied. An overall mean inhalable emission rate of 174.6 mg/h/pig and a mean internal building concentration of 1.74 mg/m^3 were measured in the piggery buildings. An overall mean emission rate of 40.1 mg/h/pig and a mean internal concentration of 0.26 mg/m^3 were measured for respirable dust. The lowest inhalable dust concentrations were measured in dry sow buildings (0.804 mg/m^3), while measurements taken in weaner buildings had the highest concentrations (2.66 mg/m^3). Straw based shelters had the highest mean respirable dust concentration (0.64 mg/m^3) and emission rate (1771.7 mg/500 kg live weight).

Key words: air quality, dust, survey, risk factors, inhalable, respirable, emission

Introduction

Generally, the airspace of intensive piggery buildings is filled with the mixture of different airborne pollutants, including airborne dust. The finer fraction of the biologically active airborne material is often referred to as 'bioaerosol', which is a complex mixture of different microorganisms and airborne particles often acting as carriers for the microbes and different gasses absorbed in them (Seedorf et al., 1998). There are essentially three major areas of concern in relation to airborne dust, such as (1) emission issues, (2) human and (3) animal health effects (Banhazi et al., 2009). High airborne dust concentrations are a concern for livestock managers as a number of studies demonstrated the association between airborne particles and different lung-related diseases in animals and humans (Donham et al., 1989; Crook et al., 1991). A number of studies have also demonstrated significant effects of sub-optimal air quality on production efficiency (Urbain et al., 1999). Therefore, the two main objectives of this study were to (1) document internal concentrations of airborne dust in different types of piggery buildings used in commercial production systems in Australia; and to (2) calculate, using refereed methodology, the emission levels of airborne dust from different types of piggery buildings in Australia.

Material and methods

In total 160 piggery buildings were included in the study. Each herd received 4 two-day visits during a period of 1 month with a different section of the farm monitored at each visit. On each farm, dry sow, weaner, grower/finisher sheds, farrowing rooms and on some farms, straw based shelters, were surveyed during the study (Banhazi et al., 2008b; Banhazi et al., 2008c). Details of the techniques used for measurement of dust concentrations have been described by other articles (Banhazi et al., 2008b; Banhazi et al., 2008c), so it is not repeated here. The estimate of emission rate was determined from the product of the ventilation rate, which was based on the carbon dioxide balance method. For predicting emission levels, the European ANIPRO (developed from the early version of "Stalkl") program was used. Carbon dioxide were monitored continuously using a Multi Gas Monitoring (MGM) machine developed in-house (Banhazi et al., 2008d). Window based STATISTICA 6.0 (StatSoft Inc., 1996) was used to conduct basic statistical manipulation of the data, such as grouping and descriptive statistics. A detailed model was later developed to test various interactions and the results of the detailed analysis have been published previously (Banhazi et al., 2008a; Banhazi et al., 2008c; Banhazi et al., 2008d). However, in this paper grouping (one-way ANOVA) was used to report on average values recorded in different buildings.

Results

The results of internal concentrations of airborne dust measured in different types of piggery buildings included in the study are shown in Table 1. The highest total airborne dust concentrations were detected in straw based shelters and weaner buildings with mean concentrations of 2.57 and 2.66 mg/m^3, respectively. In contrast to the previously mentioned buildings, houses for dry sows had lowest concentrations of inhalable dust of 0.804 mg/m^3.

Table 1 Inhalable and respirable dust concentrations(mg/m³) inside the study buildings(Summary table of means)

Building type	Mean	No. of buildings	Minimum	Maximum
		Inhalable dust		
Grower	1.678	37	0.240	4.839
Finisher	1.674	27	0.389	4.291
Straw based shelters	2.567	11	0.273	7.677
Dry sow	0.804	22	0.125	4.580
Farrowing	1.225	29	0.123	5.146
Weaner	2.657	33	0.145	10.072
All groups	1.738	159	0.123	10.072
		Respirable dust		
Grower	0.237	37	0.021	0.698
Finisher	0.305	27	0.034	1.190
Straw based shelters	0.642	11	0.121	2.130
Dry sow	0.161	22	0.033	0.397
Farrowing	0.179	29	0.014	0.468
Weaner	0.267	33	0.055	0.974
All groups	0.262	159	0.014	2.130

The respirable dust concentrations in straw based shelters were clearly very high, ranging between 0.121 and 2.13 mg/m³, with the mean value of 0.642 mg/m³. This average value is almost 3 times the recommended maximum level currently adopted in Australia as safe respirable dust levels(0.23 mg/m³) in livestock buildings (Banhazi et al., 2008b). Dry sow(0.16 mg/m³) and farrowing buildings (0.18 mg/m³) recorded the lowest means numerically as well as the lowest maximum concentrations(Table 1).

The mean inhalable dust emission was 1306.7 mg/h/500 kg live-weight(Table 2). Farrowing(509.6 mg/h/LSU) and dry sow(411.2 mg/h/LSU) buildings had very low emission rates, while straw based shelters had the highest inhalable dust emission rates calculated by far(4925.1 mg/ h/LU). Straw based shelters recorded the highest maximum emission rate of inhalable dust as well which was approximately 30 times higher than maximum emission from dry sow buildings. The mean respirable dust emission rates were again the lowest from dry sow(80.8 mg/h/LU) and farrowing buildings(60.4 mg/h/LU) and were the highest from straw based shelters(1771.7 mg/h/LU). Emission rates per animal are also presented in Table 2. Straw based shelters again recorded the highest emission rates per pigs (313.5 mg/h/animal). The overall respirable dust emission rate was 40.1 mg/h/animals.

Discussion

Overall, the concentration of respirable dust, as well as emissions of inhalable and respirable dust (as expressed per LSU or per animals) were the highest in straw based shelters. The current "safe" concentration recommendation in Australia for exposure of respirable dust is 0.23 mg/m³ and 2.4 mg/m³ for inhalable dust. The mean concentration of inhalable dust in straw-based shelters was also a concern, as it exceeded recommended levels. In terms of respirable and inhalable dust levels, Australian piggery buildings generally recorded comparable or higher levels that previously published results (Takai et al., 1998). Differences observed in concentration between the published results from Europe and the Australian study might be due to the rate of dust generation within the buildings and/or the clearance by various routes. The second highest respirable dust concentrations were measured in buildings housing finisher pigs. Relatively high concentrations of respirable dust were measured in weaner buildings, however this was not surprising. Weaner sheds are usually kept warm all year around and ventilation levels in these buildings are typically low. Weaner pigs also tend to be fairly active, creating turbulences and therefore high dust concentrations in buildings housing them. The current recommendations for acceptable respirable dust concentrations in livestock buildings is 0.23 mg/m³ in Australia (Banhazi et al., 2008b). This recommendation is not enforced by legislation, but is recommended by most housing experts in Australia. From the results of the research presented, it can be concluded that the average respirable dust concentrations measured in various piggery buildings in Australia is above the currently recommended acceptable level. The potential effects of high relatively high inhalable and respirable dust emissions from piggery buildings on the rural environment need to be considered. The per animal emissions of respirable and inhalable dust were extremely high for straw based shelters. These very high emission rates do provide for reasons for further investigations.

Table 2 Inhalable and respirable dust emission values per livestock units (LSU = 500 kg live weight) and per animal from different piggery buildings (mg/h))

Building type	Mean	No. of buildings	Minimum	Maximum
Inhalable dust				
Grower (LSU)	1041.3	28	20.3	2576.9
Finisher (LSU)	1031.1	19	212.4	2690.5
Straw based shelters (LSU)	4925.1	8	543.6	26747.0
Dry sow (LSU)	411.2	14	48.5	884.8
Farrowing (LSU)	509.6	18	122.4	1691.0
Weaner (LSU)	1788.6	22	260.8	8059.9
All groups (LSU)	1306.7	109	20.3	26747.0
Grower (per animal)	94.4	28	2.0	221.7
Finisher (per animal)	154.2	19	35.3	471.5
Straw based shelters (per animal)	787.0	8	44.0	5081.9
Dry sow (per animal)	131.7	14	17.0	309.7
Farrowing (per animal)	238.9	18	57.5	791.4
Weaner (per animal)	46.3	22	7.5	209.6
All groups (per animal)	174.6	109	2.0	5081.9
Respirable dust				
Grower (LSU)	121.5	28	4.4	418.7
Finisher (LSU)	194.4	19	37.3	510.3
Straw based shelters (LSU)	1771.7	8	67.9	12150.0
Dry sow (LSU)	80.8	14	12.8	193.3
Farrowing (LSU)	60.4	18	9.8	293.7
Weaner (LSU)	194.5	22	23.0	1942.9
All groups (LSU)	254.8	109	4.4	12150.0
Grower (per animal)	11.4	28	0.4	46.1
Finisher (per animal)	29.7	19	5.2	84.7
Straw based shelters (per animal)	313.5	8	2.8	2308.5
Dry sow (per animal)	26.0	14	4.5	67.6
Farrowing (per animal)	28.3	18	4.6	137.4
Weaner (per animal)	5.0	22	0.6	50.5
All groups (per animal)	40.1	109	0.4	2308.5

Conclusions

Concentrations and emissions of both inhalable and respirable dust were investigates in 160 Australian piggery buildings. By large straw based shelters showed the highest concentrations of respirable dust with average values of 0.642 mg/m^3. The highest emission rates of inhalable and respirable dust (per LSU and per animal) were also observed in straw based shelters. Given the recorded concentration and emission values, dust concentrations and especially emissions need to be further investigated in straw based shelters.

Acknowledgments

This study was part of a larger project funded by the Australian Pork Limited. It was also a collaborative effort between the South Australian Research and Development Institute (SARDI), Agriculture Western Australia, The Queensland based Pig Unit and Agriculture Victoria and involved the contribution of many people. We wish to particularly acknowledge the contribution of pig producers involved in the study, Dr Colin Cargill for his professional advice and the assistance of all technicians involved in the study.

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Microarray Analysis of Gene Expression in Rams Experimentally Infected with the Rough Virulent Strain of *Brucella ovis*

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Summary: The analysis of gene expression profiles was characterized in rams experimentally infected with the rough virulent strain of *Brucella ovis* (R-*B. ovis*) trough microarray analysis. The results reported herein represent the first microarray analysis performed on tissues of rams infected with R – *B. ovis*.

Introduction

Brucella ovis is the causative agent of ovine brucellosis or contagious epididymitis, a disease mainly characterized by infertility in rams(Megid et al., 2010). *Brucella* spp. replicate and persist within macrophages in host tissues, including male and female reproductive tissues(Moreno and Gorvel, 2004). Infection of these tissues with *B. ovis* results in a moderate inflammatory response, and virulence factors promote evasion of the immune response favoring persistent infection (Xavier et al., 2011, Antunes et al., 2013). In this study, characterization of gene expression using total mRNA was done by microarray hybridization in multiple tissues collected over of eight months dpi from rams experimentally-infected with R – *B. ovis*.

Material and methods

The microarray analysis of each tissue was done at three collection times: acute infection(60 days post infection [dpi]), chronic infection phase I(120 dpi), and chronic infection phase II(240 dpi), and the tissues studied included reproductive organs(epididymus, testicles, ampolae, vesicular glands, bulbourethral glands) and a pool of lymph nodes(inguinal and scrotal). The gene expression profiles associated with R-*B. ovis* infection were determined using the Affymetrix Bovine Genome Array and expression levels of infected and non infected rams(control group, 0 dpi) were compared.

Results and discussion

Of the 23,000 genes analyzed in the microarray, differentially expressed genes(DEGs) included 139 during acute phase of infection, 930 during the chronic phase I and 744 during the chronic phase II of infection. Thirty known genes and fourteen unknown genes were expressed during the three phases of infection. The biological functions of these genes included immune cell trafficking, immunological disease, infectious disease, inflammatory disease, inflammatory response and cellular movement; and significant differences were observed at the three phases of infection.

Conclusions

The results reported herein represent the first microarray analysis performed on tissues of rams infected with R-*B. ovis*. These results expand the pathogenesis knowledge of this R-*B. ovis* strain infection in rams and suggest new genes and pathways to be investigated in the future.

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Effects of Ciprofloxacin on the Carbon-source Metabolism Function of Soil Microbial Communities

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Abstract: Ciprofloxacin (CIP) has been extensively applied to treat kinds of infectious diseases in human and animals. To evaluate the effects of CIP residues on the function of microbial community in soil, the carbon-source metabolic functions of the soil microbial communities exposed to CIP were studied by the method of BIOLOG. The results showed that the carbon-source metabolic capacity and diversity of the soil microbial communities exposed to 0.1 μg/g, 1 μg/g, 10 μg/g and 100 μg/g CIP were significantly influenced, in different patterns. I. e., the carbon-source metabolic functions of the soil microbial communities exposed to 0.1 μg/g, 1 μg/g and 10 μg/g CIP for day 7 and 21 were significantly lowered but no significant difference found after day 35, while the metabolic functions of the soil microbial communities exposed to 100 μg/g CIP for day 7, 21, and 35 were all significantly lowered. It was concluded that a certain accumulation of CIP in soil, for instance, 100 μg/g CIP performed irreversible and long-term effects on the metabolic functions of the soil microbial communities.

Use of Sodium Acetate in the Treatment of Piglets' Post—Weaning Colidiarrhea

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Abstract: The goal of these trials was to create the optimal scheme of colidiarrhea treatment weaned piglets using ecologically pure and cheap medicine.

One of the main causes of this disease is achlorhydria which destroys the organism natural barrier against environmental micro flora ingested with the feed. Moreover, weaning, absence of "milk defense", forming new groups of animals provoke the so called post-weaning stress which results in activation of sympathetico-adrenal system and achlorhydria. That is why we decided to find the substance which stimulates the luminal glands and normalizes gastric acid secretion and in that way to stop the colonization of small intestine with the environmental micro flora. This substance is very convenient to be sodium acetate.

Trials were conducted at the JS "Akhtubinets" swine breeding farm during a mass colidiarrhoea outbreak. Diagnosis was affirmed in the local veterinary laboratory. The strain of *E. coli* O141:K88 was identified in the intestine of dead piglets. Treatment was carried out with sodium acetate (JS "Khimprom"). Fifty piglets each were assigned to: control and experimental groups (CG and CC). Piglets were 40 – 45 days old and were kept in the adjacent pens. Feeding and maintenance conditions were equal for all animals. Experimental piglets were given 5 ml 3% aqueous solution of sodium acetate per os from a syringe cannula in 40 – 60 min before each prandial feeding for 10 days. Control piglets were treated with tylosine IM according to the manufacturer's instructions (the identified strain of *E. coli* was not acceptible to any antibiotics). Only one piglet died in the EG whereas in the CC four and four animals continue to sick.

Quantity of the "diarrheic" days in EG was 26 that on 84. 6% less, then in CC(48). Body weight gain in EG was 1. 5 ± 0. 4 kg, in CC – 0. 7 ± 0. 2 kg, i. e. on 114. 3% less ($P < 0.04$).

Formation of Chosen Blood Parameters of Hy-Line Laying Hens in Different Egg-laying Phases

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Abstract: Formation of blood parameters is dependent on the physiological condition of organism, patogen factors, breeding technology and type of animal feeding. Especially in intensive rearing of laying hens the evaluation of biochemical blood characteristics is very important because it shows the level of animals welfare. The problem is significant due to current lack of reference intervals for these blood parameters which are very variable depending on the ageand egg-laying phase.

The research aims to analyze chosen biochemical blood parameters of Hy-Line laying hens and determine their reference intervals according to egg-lying phases. Birds were housed in a battery system and fed complete feed mixture designed to meet nutritional needs of laying hens concerning energy and protein in different egg-laying phases. Blood samples were collected from a brachial vein of clinically healthy birds which were between 21 – 65 weeks of age. In blood serum the fallowing numbers of samples for each parameter were marked: albumin(Alb), 1225; total protein(TP), 1420; glucose(G), 1113; urea(U), 831; uric acid(UA), 982; creatinine(C), 663; alanine aminotransferase(ALT), 1265; aspartate aminotransferase(AST), 1277 and alkaline phosphatase(AP), 950. Analyses were performed in laboratory included in international quality control system RIQAS(Randox International Quality Assessment Scheme). All the above analyses were conducted using biochemical analyzer Pentra 400 and commercial kits produced by Horiba ABX Company.

All the results were analysed statistically using SAS software. In consequence, the reference intervals for chosen biochemical blood parameters of laying hens according to egg-lying phases were determined.

The Effect of Intermitent Photic Stimulation on Sheep Welfare

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Abstract: The paper presents a stress reaction in sheep as a result of the high-frequency light effects with a strong impact on experimental animals, reducing welfare. The application of intermittent photic stimultion(IPS) is a usefulness method as a causative agent of photoparoxysmal response of the cerebral cortex in EEG and a common practice in human medicine. Mentioned method was used as an artificial, experimental factor in three groups of rams: control(Ⅰ) and two groups tested with IPS at stimulation level of 1 Hz(Ⅱ) and 10 Hz(Ⅲ), resulting in the stress response of the animal body. Bioelectrical activity of the cerebral cortex divergences as a response to given stimulus, may reveal in the EEG as a single spike wave up to the epileptic discharges. The EEG studies in sheep showed a harmonic response of central nervous system in multiple stimulation of a frequency of 1 Hz. It was found that the sheep cerebral cortex was characterized by cyclic burn-suppression patter during experimental photostimulation at 10 Hz. The EEG record is quite difficult to interpret, because the phenomenon of burst-suppression patter is associated with sudden, short-term and often high voltage potentials. The experimental protocol included also the examinations of physiological and endocrine parameters in animals. It was found that the mean HR/min. in sheep during the photostimulation was 90. 34, RR/min. 57. 91(Ⅰ), 69. 11(Ⅱ) and 75. 00(Ⅲ) with significant differences($P < 0.001$). The mean cortisol level was 1. 77 ng/ml(Ⅰ), 3. 76 ng/ml(Ⅱ) and 14. 24 ng/ml(Ⅲ) with significant differences($P < 0.001$). It was also found that the differences between the adrenocorticotropic hormone(ACTH) levels were statistically significant($P < 0.05$): 15. 13 ng/ml(Ⅰ) up to 404. 2 ng/ml(Ⅲ). During the study the adrenaline and noradreanline presence were also monitored with similar growth trends.

Radiation Monitoring in Soils of the City of Volgograd

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Abstract: In article the radiating situation the city of Volgograd of the Russian Federation is investigated. In communication with what radiating inspection and control of a radiating situation (considering the importance of low powers of doses on the besporogovy mechanism in risk of emergence of the remote consequences of radiation) is given importance in the solution of problems of health and safety of the population and protection of environment. The Volgograd region from the South, the North and the West borders on the Rostov, Voronezh, Saratov areas where dangerous objects (the Volgodonsk, Balakovsky, New Voronezh nuclear power plant) are located radiatsionno.

The purpose of researches was studying and an assessment of a radiating situation in the territory of the city of Volgograd.

As a main goal of the real work was definition of a radiating situation in the territory of Volgograd us the following methods were chosen:

1. district gamma shooting;
2. sampling of objects of control with the subsequent spectrometer and radiometric analysis.

Proceeding from results of the carried-out works, the conclusion is drawn on existence in почвогрунтах traces of a radionuclide of Caesium-137 (less than 1 Ki/km^2) owing to Chernobyl failure, instead of emissions from the nuclear power plants located in the Rostov, Saratov, Voronezh areas.

In test of the soil which has been selected in Dzerzhinsk the area the "dot" sites having the raised radiating background are found. The capacity of an effective dose on a surface почвогрунтов made from 0,4 to 0,6 мкЗв/hour that above intervention level (0. 2 mkzv/hour). The spectrometer analysis of tests showed existence of a technogenic radionuclide Caesium-137, with specific activity 2649 ± 280 to Bq/kg according to Standards of radiating safety (NRB-99/2009), quantity of the found radionuclide Caesium-137 doesn't exceed minimum significant specific activity of MZUA (10000 Bq), and doesn't demand additional intervention.

In the Voroshilovsky area in soil test, values of specific aktivnost of radionuclides Potassium – 40 – 632ü¬ /kg, Radium – 226 – 34ü¬ /kg and Thorium – 232 – 33ü¬ /kg the soils raised in comparison with another sites are found. MED on this site was also raised concerning other sites and made value 0,2 мкЗв /h that exceeded MED on nearby sites (0. 08 mzv/h) though also is radiatsionno safe. The reason of the raised maintenance of natural radionuclides (ERN), can be clay - enriched with organic inclusions of tumors of phosphorites and layers of the uglefitsirovanny remains of the plants, containing ERN.

On the basis of the carried-out researches the conclusion is drawn that the situation existing now (in the territory of the surveyed districts of Volgograd) is estimated as radiating and safe.

Economic Analysis on HPAI Prevention and Control Strategies Optimization and the Threshold of Vaccination Withdrawal in Southwest China

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Abstract: HPAI prevention and control involves the sustainable development of poultry husbandry, exportation, increasing farmer's income, public health and national security. Vaccination can effectively decrease incidence. However, each vaccination campaign should have an exit strategy since the high cost of vaccination can prove an obstacle or a drawback of a long-term large scale vaccination programme. In this project, lowest-cost method was proposed to animal disease prevention and control alternatives optimization and the threshold calculation of vaccination withdrawal to avoid the difficulty of benefit evaluation, assuming the result of doing-nothing was disease outbreak and different interventions would come to the same result of stamping out the disease. Taking a province in southwest China as an example, poultry HPAI between-herd spread model was established by applying the North American Animal Disease Spread Model (NAADSM). Three alternatives, "mass vaccination + culling", "stop vaccination + culling" and "stop vaccination + improved culling" were simulated and epidemiology parameters, the output of the model, were added into economic models to estimate the cost of alternatives. The results showed that shortening the first detection time and improved culling (reducing radius of destruction ring) were the key factors to reduce prevention and control cost of HPAI. The 30 years technical economics analysis on the alternatives showed that, "stop vaccination + improved culling" would be the optimal strategy in the long term, and the threshold of stopping vaccination is poultry individual prevalence below 0.09%, with the precondition of shortening the first detection time to 6 days, and applying improved culling and strict movement control in disease zones and threaten zones. However, the result of the technical economics analysis only provided a reference for decision-making, as social institution and political factors were not considered, and migratory bird factor was not involved in the poultry HPAI between-herd spread model.

Drug Resistance and Recent Therapeutic Measures in Controlling of Fascioliasis

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Abstract: Fascioliasis is a widely distributed disease affecting herbivorous animals. As a result of drug resistance a mixture of two antifasciola drugs (Triclobendazole and Superivomec) was used in trial to overcome this drug resistance. Twenty eight newly weaned white Boskat rabbit aging 1.5 month were divided into 7 groups, six of them were experimentally infected with metacercaria of *Fasciola gigantica* and one kept as ve control group. Faecal egg count during the clinical course of the disease, counting the worm and its morphological studies and lesion score after postmortem examination were the parameters used to evaluate the effect of different drug mixtures. It had been concluded that the mixture of triclabendazole and superivomec was the mixture of choice.

Key words: fascioliasis, metacercaria, triclabendazole, rabbit and superivomec.

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