SEROLOGICAL SURVEY OF ANTIBODIES AGAINST *TOXOPLASMA GONDII* IN ORGANIC SHEEP AND GOAT FARMS IN GREECE

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SUMMARY

In the present study, a preliminary and a main research for IgG antibodies against *Toxoplasma gondii* were conducted in organic sheep and goat farms of Western Greece, by means of ELISA. In the preliminary research, 413 sera were tested and the significant resulting seroprevalence triggered further research that was conducted one year later. For the main research, 349 sera were tested. Results indicated that seroprevalence against *Toxoplasma gondii* is high in organic farms, both in animals that had or had not aborted, is more prevalent in older animals and susceptibility alters between gender and species.

Keywords: *Toxoplasma gondii*, sheep, goats, organic, ELISA, Greece

INTRODUCTION

Toxoplasmosis is a parasitic zoonosis caused by the protozoon *Toxoplasma gondii*, an intracellular parasite which infects a wide range of animal species. Over two hundred of terrestrial and marine mammals (Haralambidis 1995, Dubey *et al*. 2003), along with birds (Dubey 2002) and human seem to be intermediate hosts of this parasite. Members of the family Felidae, such as domestic cat, act as definite hosts (Urquhart *et al*. 1996).

Sporulated oocysts, bradyzoites encysted in tissue and tachyzoites are the three infectious stages in the life cycle of *T. gondii*. These stages are infectious for both intermediate and definite hosts (Bowman 1999).

Herbivores are mainly infected by oral ingestion of sporulated oocysts (Jubb *et al*. 1993), which are very resistant to environmental conditions (Tenter *et al*. 2000). In intermediate hosts, such as sheep and goats, primary exposure to *T. gondii* during gestation can lead to vertical transmission of toxoplasmosis that occur transplacentally to the fetus (Tenter *et al*. 2000) and it is believed that may occur more than once and in successive litters (Buxton *et al*. 2006, Duncanson *et al*. 2001). In several species, such as sheep, goats and cattle, *T. gondii* transmission occurs after consumption of tachyzoites' contaminated milk (Powell *et al*. 2001). In sheep, venereal transmission is also probable (Martinez-Garcia *et al*. 1996). In all cases, *T. gondii* forms tissue cysts after host infection (Bowman 1999).

Toxoplasmosis in sheep is mainly asymptomatic, except for the occasional fever and tachypnea during the first days post infection (Esteban-Redondo *et al*. 1999). Goats however and especially kids can develop anorexia, diarrhoea, dyspnoea and even death when they are infected with oocysts (Dubey 1989). Fetal deaths, mummifications, abortions and birth of stillborn or weak lambs and kids are the most frequent symptoms in flocks of sheep and goats, being infected with *T. gondii*. Abortions are more frequent after mid-gestation (Esteban-Redondo and Innes 1997).

Till today toxoplasmosis research carried out in Greece, referred only to conventional sheep and goat farms (Haralabidis 1987, Stefanakis *et al*. 1995, Kontos *et al*. 2001, Diakou *et al*. 2005a, Diakou *et al*. 2005b) and no data are available for the organic farming, which is a strongly developed sector.

The aim of the present study was to detect antibodies against *T. gondii* in organic flocks of sheep and goats at Western Greece and to evaluate the parasite's potential role in the manifested abortions.

MATERIALS AND METHODS

Sampling

The present study was conducted in two phases: preliminary and main. For the preliminary research, in 2005, blood samples were collected from 413 animals (47 ewes and 116 goats that aborted during the reproductive period of 2004–2005, 137 rams and 113 male goats) from 11 sheep, 8 goat and 8 mixed organic flocks of Peloponnese and Western Sterea Hellas and antibodies against *T. gondii* were detected.

For the main research, in 2006, blood samples were collected from 349 animals (23 ewes and 49 goats that aborted during the reproductive period of 2005–2006, 107 ewes and 85 goats that did not abort, 52 rams and 33 male goats) from 7 sheep, 4 goat and 2 mixed organic flocks of Peloponnese, Western Sterea Hellas and Ioannina County. All samples were categorized into three groups: adult males, females that aborted during the reproductive period 2005–2006 and females that did not abort during the same reproductive period. The first two groups are consisted of relatively few animals while the rest animal population is the largest. For this reason, the sample size per flock was chosen to represent 25% of total males and females that aborted and 7% of females that did not abort.

Blood was collected from the jugular vein using vacutainer tubes without anticoagulant. Blood samples were placed in cryo-boxes and were transported to the lab within 8 hours. Serum was collected after centrifugation (2000 *rpm* for 20 minutes) in 1,5ml vials and kept frozen at –20ºC until tested.

Antibodies detection

T. gondii specific IgG antibodies were detected by means of enzyme-linked immunosorbent assay (ELISA). The assay was completed in four stages (Haralabidis 1984). Antigen (*in vivo* cultured parasites) was diluted in a buffer solution ($NaHCO_3$–Na_2CO_3, pH 9.6) and it was added in flat bottom, polystyrene microtiter plate of 96 wells. After a two hour incubation in room temperature and washing of the microtiter plate with distilled water containing 0.05% Tween-20, animal sera diluted 1:300 in PBS, pH 7.2, were added in the microtiter plate. Three pairs of control sera in each plate were used. After incubating and washing the microtiter plate as before, during the third stage, the conjugate was added: Anti-Sheep IgG, whole molecule Sigma A-5187 (diluted with

PBS, pH 7.2) was used for sheep sera and Anti-Goat IgG, whole molecule Sigma A-4187 (diluted with PBS, pH 7.2) was used for goat sera. During the fourth stage, after incubation and washing of the microtiter plate, enzyme substrate (p-Nitrophenyl Phosphate, Sigma 104 Phosphate Substrate), diluted in buffer solution ($NaHCO_3$–Na_2CO_3, pH 9.8) was added. After a ten minute incubation, stop solution, 0.1–3N NaOH was placed in every microtiter plate well and test results were being analyzed using a photometer (HUMANREADER, HUMAN Diagnostic Systems, Germany) at 405 nm wave length.

Results were estimated after the determination of the cut off value. Cut off value was determined by adding to the average of the optical density (OD) of negative control sera values their threefold standard deviation:

$$\text{Cut off} = \text{Ave} + 3\text{SD}$$

Serum samples with OD values, higher than the cut off were considered to be positive, whereas serum samples with OD values lower than or equal to the cut off were considered to be negative (Haralabidis 1987).

Statistical Analysis

Statgraphics Plus 4.0 statistical package was used in the comparison of proportions. P-values<0.05 were considered significant at 5% confidence level.

RESULTS

In the preliminary research, in sheep, 63 (45.99%) out of 137 rams and 33 (70.21%) out of 47 ewes that aborted were positive and in goats 22 (19.47%) out of 113 male goats and 29 (25%) out of 116 female goats were positive.

In the main research, in sheep, 20 (38.46%) out of 52 rams, 14 (60.87%) out of 23 ewes that aborted and 58 (54.21%) out of 107 ewes that did not abort were positive. In goats, 4 (12.12%) out of 33 male goats, 7 (14.29%) out of 49 goats that aborted and 19 (22.35%) out of 85 that did not abort were positive.

In sheep, rams had the lowest seroprevalence and ewes that aborted the highest. Ewes of both groups had significantly higher seroprevalence than the rams ($p<0.05$). The difference in seroprevalence between the two female groups was not statistically significant ($p>0.05$).

In goats, the lowest seroprevalence was that of the males, though there was no statistically significant difference between the three groups ($p>0.05$). Seroprevalence in sampled goats was significantly lower than that in sheep in all three groups ($p<0.05$).

In addition, based on age criteria, sheep and goats were categorized in two age groups of 0–4 years old and older than 4 years. In sheep, seroprevalence for animals of 0–4 years old was 39.85% and for animals older than 4 years was 61.97%. In goats, seroprevalence for the same age groups was 13.04% and 24% respectively. Seroprevalence in both species increased significantly with age ($p<0.05$).

DISCUSSION

In Greece, organic sheep and goat farming is of major economic importance and it possesses the largest sharing portion of all farmed animals (79% of the total, with 49% attributed to goats and 30% to sheep) (Ministry of Rural Development and Food, 2004). Sheep and goats get infected with *T.gondii* mostly with oocysts that are excreted in the environment via cat faeces (Urquhart et *al*. 1996). Cats are mainly used for the elimination of rodents and have access to animal storage rooms where feedstuffs can get contaminated by their faeces. In some cases, infected cats do not stay in the farm but are located in the overall area and they have occasional access to feedstuff storage rooms.

The preliminary research revealed for the first time that in organic sheep and goat farms of Greece (Peloponnese and Western Sterea Hellas) a significant number of animals are infected with *T. gondii*, a zooanthroponotic pathogen. It was surprising that animals tested one year after they had aborted, still had a high antibody titre indicating the strong immune response to the parasite. Difference in seroprevalence observed between sheep and goats and between males and females (Innes 1997, Roberts *et al*. 2001), further triggered us for the main research.

The main research revealed that the comparison of the proportions of the seropositive female animals that had abortions during reproductive period 2005–2006 and the female that did not have abortions during the same reproductive period did not have significant differences both in sheep and goats. The disease seems to be enzootic among farms and for this reason it is not expressed with massive abortions. Evaluating the relation between animal sex and difference in susceptibility against *T. gondii,* males have lower proportion of seropositive animals compared to the results of the female animals in both species. Between the two different species tested, the results are significant only in sheep. Female animals are more susceptible compared to males, because of their hormone profile (Roberts *et al*. 2001). This is due to the effect of estradiol and progesterone, on non specific (Walker *et al*. 1997) and specific immunity against *T. gondii* (Roberts *et al*. 1995).

The main research also suggests that goats have significantly lower proportion of seropositive animals in all three groups compared to sheep. Those results are in agreement with those mentioned in similar surveys (Tenter *et al*. 2000, Van der Puije *et al*. 2000) and can be explained by the difference in susceptibility among the intermediate hosts of *T. gondii.* Some scientists believe that different species susceptibility is related to how quickly the immune system can produce the key cytokine IFNγ and mention that goats are more resistant than sheep concerning infection of the parasite (Innes 1997). Goats that are reared in those organic farms seem to be more susceptible than sheep. However, some other researchers demonstrate that goats have more severe clinical symptoms of toxoplasmosis that can be result to death (Dubey 1989, Kaufmann 1996). If the above statement is correct, the higher mortality of goats compared to sheep may keep the proportion of seropositive animals at lower levels.

The proportion of *T. gondii* seropositive animals increases with age in both sheep and goats. This can be attributed to the fact age increases the possibility of infection. Taking into consideration that toxoplasmosis is a chronic disease (Esteban-Redondo and Innes 1997) and that infected sheep and goats are seropositive for a long lasting period (Conde *et al*. 2001) it is expected that older animals have higher proportions of seropositive individuals. It can also be noted that seroprevalence in those organic farms is high enough to conclude that the disease is enzootic. In all cases, special attention should be given to the storage of organic feedstuffs and management of organic pasture, in order to prevent their contamination by infected Felidae.

In conclusion, the results of the present research justify the presence of IgG antibodies against *T gondii* in organic sheep and goat farms in Western Greece. They also demonstrate that the disease in these farms is enzootic while there is different susceptibility between goat and sheep and between sexes. Further research is needed to clarify the proportion of sheep and goats that have antibodies against *T. gondii* in the total number of organic farms in Greece and also to reveal the different susceptibility among those species.

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