EFFECTS OF LAMB SUCKING ON THE BACTERIAL FLORA OF TEAT DUCT AND MAMMARY GLAND OF EWES

Gougoulis, D.A., Kyriazakis, I., Tzora, A., Taitzoglou, I.A., Skoufos, J. and Fthenakis, G.C.

Veterinary Faculty, University of Thessaly, 43100 Karditsa, Greece

ABSTRACT

Objective. We determined differences in bacterial flora populations of teat duct and mammary gland of ewes before and after sucking by lambs; we also evaluated factors potentially affecting these differences. Methods. We collected samples of teat duct material (by means of fine [20 G], plastic, sterile 2 mm-long catheters) and mammary secretion from 11 ewes immediately before (< 40 s) and immediately after (<30 s) sucking by their lambs, as well as 120 min later. We processed samples by conventional bacteriological techniques. We compared changes in infection by means of Sign Test. Results. We isolated bacteria from 3.5% duct and 1.5% secretion samples before sucking; respective figures after it were 10.6% and 2.0%, and 120 min later 6.8% and 1.5%. We saw differences among duct material samples collected before and after sucking, in 40 cases: from 6 bacteria were isolated only before, from 34 only after (p<0.001); respective results for secretion were 4 cases. We saw differences among duct material samples collected after suckling and 120 min later, in 12 cases: 8 and 4 (p=0.375), respectively; no changes were for secretion samples. We found neither difference among ewes with single or twin lambs, nor among stages of lactation. Mostly, we isolated staphylococci: 70% of isolates before suckling, 80% of isolates after it, 91% of isolates 120 min later; we also isolated two *Mannheimia haemolytica* strains immediately after sucking. Conclusion. Sucking predisposes to entrance of bacteria into the teat duct; however, increased teat duct infections did not result to respective mammary infections. Subsequently, teats overcame the infections. *M. haemolytica* isolated directly after suckling indicates lambs as source of infection.

INTRODUCTION

It has been repeatedly in studies of ovine mastitis, that sucking by lambs is associated with transfer of microorganisms into the teat duct [12, 19, 22]. There are three different sources of bacteria, which might enter into the teat duct, namely the lamb (mouth, nasopharynx), the ewe (udder skin) or the environment (bedding). These organisms may subsequently ascent to the mammary gland and cause mastitis. The objectives of this investigation were i) to determine differences in bacterial flora populations of teat duct and mammary gland of ewes before and after sucking and ii) to evaluate factors that potentially affect them.

MATERIALS AND METHODS

Sampling schedules

Eleven multiparous Karagouniko-breed lactating ewes were included in this study within 5 days after lambing and housed separately throughout. Ewes were selected among animals with no history of mastitis; the California Mastitis Test (CMT) was performed in mammary secretion samples from both glands of these ewes, with five degrees of reaction scores ("negative", "trace", "1", "2", "3") as detailed by Fthenakis [9]. Teat duct material and mammary secretion samples were obtained and processed as detailed below. From the ewes selected, seven suckled a single lamb and four suckled twins. Standard husbandry conditions applied throughout the study. The work was carried out under a license for experimental procedures issued by the Greek Ministry of Agriculture, based on EU guidelines. Paired-samples were obtained from both mammary glands of each ewe. Samples were obtained on three occasions weekly, for six weeks (2nd to 7th of lactation). Lambs were separated from their dams for 60 (±3) min; during that time no feed, milk or water were provided. Samples ("A") were collected and within 30 s lambs joined their dams; they immediately (<7 s) started sucking, whilst ewes were restrained in the standing position. Then new samples ("B") were collected, by following a different schedule on each of the sampling occasions. For collection of samples "B_1" (1st sampling occasion of each week), natural termination (i.e., by the lamb or the ewe, without human interference) of a sucking bout was allowed. For collection of samples "B_2" (2nd sampling occasion of each week), lambs were removed from their dam 3 min (±2 s) after joining her. For collection of samples "B_3" (3rd sampling occasion of each week), lambs were removed from their dam 1 min (±2 s) after joining her. In all cases, samples were obtained within 30 s. All lambs were observed, to confirm that both teats of the dam had been sucked (including ewes with a single lamb). Finally, further samples ("C") were collected 120 (±5) min after collection of "B_1" samples.

Collection and processing of samples

Before sampling, a thorough clinical examination was carried out on the ewes, with special attention paid to their mammary glands and teats [8, 19]. A thorough disinfection with povidone iodine scrub solution was carried out in the teat apex and the lower (1 cm) part of the teat skin. A sterile, plastic, 20 G catheter (Abbocath®; Abbott Laboratories Inc., Abbott Park, USA) was used for sampling material from the teat duct. The catheter stylet was taken out and the plastic catheter was cut with a sterile blade to a length of 2 mm. In order to ensure accurate and consistent cutting of the catheter at the desired length, a sterilized ruler was always placed beside the catheter. The whole procedure was carried out under aseptic conditions. The catheter was held by the investigator from the cannula hub; it was inserted into the teat, rolled around the internal teat wall, in order to sample the mucosa, and then withdrawn. Description and validation details of the method were presented by Mavrogianni and others [16]. Subsequently, secretion samples were obtained. The first two squirts were drawn onto the palm of the gloved hand of the investigator and examined for the presence of abnormalities; then, 10 to 15 ml were carefully collected into a sterile container. These procedures were carried out in all samplings. Standard procedures for sampling and processing of samples previously described in detail [8, 19] have been used during the study. Duct material collected on the tip of the catheter and mammary secretion samples were plated onto Columbia 5% blood agar; the media were incubated aerobically at 37 ^{o}C for up to 72 h. Throughout this study, all bacteria isolated were identified by using conventional techniques [2,

5]; for staphylococcal identification, the "API-Staph SYSTEM" quick identification strips were also used (BioMerieux S.A., Marcy-l'-Etoile, France).

Data management and statistical analysis

The model described in a previous experimental work using paired-samples [18] was employed. Analysis of results was carried out by comparing changes in infection status between "A" and "B" samples and between "B_1" and "C" samples. Duct material and secretion samples were assessed separately. Statistical significance was assessed by the Sign Test, which allowed for the readings to be paired. Furthermore, comparisons were performed between ewes suckling single or twin lambs, as well as between "B_1", "B_2" and "B_3" samples. Finally, changes between stages of lactation (Stage I: 2nd and 3rd week of lactation, Stage II: 4th and 5th week, Stage III: 6th and 7th week) were evaluated. Analysis of Variance for proportions over time was employed. Data were modelled in Minitab 14 (Minitab Inc., State College, PA, USA). The critical probability was set at $p = 0.05$, on a 2-sided null hypothesis of no difference.

RESULTS

Clinical findings

None of the ewes included in the study had a history of mastitis. No bacteria were isolated from any duct material or mammary secretion samples obtained from the ewes before inclusion into the study. All CMT results were negative. Neither changes in mammary secretion, nor mammary abnormalities were detected in ewes sampled during the study. In all cases, lambs started sucking immediately (<7 s) after joining their dam. Both teats of the ewes were sucked. In total, 924 duct material and 924 secretion samples were collected during the study. These were as follows: 396 "A" samples (252 from ewes with a single, 144 from ewes with twins) and 132 each of "B_1", "B_2", "B_3" or "C" samples (in each of these, 84 from ewes with a single, 48 from ewes with twins).

Effects of suckling on bacterial isolations

Among "A" samples, 14/396 (3.5%) duct material and 6/396 (1.5%) secretion were bacteriologically positive. Among "B" samples, 42/396 (10.6%) duct material and 8/396 (2.0%) secretion were bacteriologically positive. Among "C" samples, 9/132 (6.8%) duct material and 2/132 (1.5%) secretion were bacteriologically positive (Table 1).

After suckling, there was a significant increase by 200% (from 14 to 42) in infected teat ducts ($p< 0.001$). No effect was found on mammary secretion: from 6 infected samples to 8 ($p=0.590$). In 40 (10.1%) cases, there was a change of bacteriological status of duct material samples in-between suckling; in 6 cases a positive sample became negative, whilst in 34 cases a negative sample became positive. Changes concerned 12/132 "A" to "B_1", 15/132 "A" to "B_2" and 13/132 "A" to "B_3" pairs of samples ($p>0.540$). Changes were observed in 22/252 pairs from ewes with singles and 18/144 from ewes with twins ($p=0.346$). They were observed in 12/132 pairs from Stage I of lactation, in 12/132 from Stage II and in 16/132 from Stage III ($p>0.420$). In 4 (1.0%) cases, there was a change of bacteriological status of secretion samples in-between suckling; in 1 case a positive sample became negative, whilst in 3 cases a negative sample became positive. Subsequently (120 min after suckling), there was a decrease by 31% (from 13 to 9) of infected

teat ducts (p=0.375). No change was recorded in mammary secretion; 2 infected samples on both occasions (p=1.000). In 12 (9.1%) cases, there was a change of bacteriological status of duct material samples during the 120 min after suckling; in 8 cases a positive sample became negative, whilst in 4 cases a negative sample became positive. Changes were observed in 7/84 paired-samples from ewes with singles and 5/48 from ewes with twins (p=0.700). Changes concerned 3/44 pairs from Stage I of lactation, 5/44 from Stage II and 4/44 from Stage III (p>0.460).

Table 1. Bacteriological status of samples from ewes before and after suckling of lambs, classified according to number of suckling lambs or to stage of lactation

	Ewes with a single	Ewes with twins	Stage I[a]	Stage II	Stage III	Total
"A" Samples (before suckling)						
Duct samples	5/252[b]	9/144	3/132	5/132	6/132	14/396
Milk samples	3/252	3/144	3/132	2/132	1/132	6/396
"B" Samples (after suckling)						
Duct samples	21/252	21/144	13/132	13/132	16/132	42/396
Milk samples	4/252	4/252	3/132	3/132	2/132	8/396
"C" Samples (120 min later)						
Duct samples	5/84	4/48	2/44	3/44	4/44	9/132
Milk samples	1/84	1/48	2/44	0/44	0/44	2/132

[a] Stage I: 2nd and 3rd week of lactation, Stage II: 4th and 5th week, Stage III: 6th and 7th week.
[b] n/m = bacteriologically positive results out of total samples.

Bacterial identity

Bacteria were always isolated in pure culture. The majority of isolates were coagulase-negative staphylococci (*Staphylococcus epidermidis*, *S. simulans*, *S. xylosus*, *S. chromogenes*, *S. sciuri*, *S. caprae*, *S. schleiferi*). These organisms accounted for 52/65 (80%) and 11/16 (69%) of the total isolates obtained from duct material and secretion samples, respectively. Other organisms isolated were: streptococci, *Escherichia coli*, *Bacillus* spp., *Mannheimia haemolytica*, *Arcanobacterium pyogenes*, *Klebsiella* sp. and *S. aureus* (Table 2).

Both *M. haemolytica* strains were isolated from duct material samples obtained after suckling (a "B_2" and a "B_3" sample) from two different ewes; both strains were isolated during the 3rd week of lactation. There were no significant differences in the proportion of staphylococci recovered before suckling (70% of total isolates), after suckling (80% of total isolates) or 120 min later (91% of total isolates) (p>0.290). Of the 16 bacteriologically positive secretion samples obtained during the study, in 11 (69%) the same organisms as those from the respective duct material sample, were isolated.

Table 2. Frequency of isolation of each bacterial species from duct material or mammary secretion samples collected from ewes

	"A" samples		"B_1" samples		"B_2" samples		"B_3" samples		"C" samples		Total samples	
	D[a]	S[a]	D	S	D	S	D	S	D	S	D	S
Coagulaseve staphylococci	11	3	10	1	13	3	10	2	8	2	52	11
Streptococci	1	2	0	1	0	1	0	0	0	0	1	4
E. coli	1	0	0	0	1	0	1	0	1	0	4	0
Bacillus spp.	0	0	1	0	2	0	0	0	0	0	3	0
M. haemolytica	0	0	0	0	1	0	1	0	0	0	2	0
A. pyogenes	0	1	1	0	0	0	0	0	0	0	1	1
Klebsiella sp.	1	0	0	0	0	0	0	0	0	0	1	0
S. aureus	0	0	1	0	0	0	0	0	0	0	1	0
Total	14	6	13	2	17	4	12	2	9	2	65	16

[a] D: duct material samples, S: secretion samples.

DISCUSSION

The experimental model

In previous studies of ovine mastitis, it has been confirmed that the teat is the portal of entry of the causal agents, the most important of which are *Staphylococcus* spp. and *M. haemolytica*, together accounting for over 80% of isolates [4]. Staphylococci are considered to originate from milkers' hands or from the skin of the udder [3, 15]). Scott and Jones [22] and Jones and Watkins [12] proposed that *M. haemolytica* originated from the tonsils of the sucking lambs; however, the hypothesis has never been confirmed. In cows, it has been established that improper milking technique predisposes animals to mastitis. As the teat canal dilates, bacteria can invade into the teat. Its orifice may remain open for up to two hours after completion of milking [14, 26], thus facilitating invasion of bacteria into the teat and subsequent ascent to the mammary gland. Similar findings have been presented in dairy ewes after hand-milking [18]. To the best of our knowledge, the possible role of lamb sucking in transferring bacteria into the teat has not been studied. The experimental model that we used, i.e. paired-samples immediately before and immediately after suckling, minimized the time in-between sampling/suckling/sampling. Thus, it ensured that bacterial isolations reflected true dynamics of infection throughout.

Dynamics of infection

We found significantly increased teat duct infections after suckling, but no strong evidence of increased intramammary infections. Infection of the teat occurred as soon as 1 min after initiation of suckling. However, there were no significant differences among the three procedures evaluated ("B_1", "B_2", B_3"). We should also consider the possibility that some bacteria enter into the teat, but are subsequently withdrawn during the same sucking. In a previous experimental study [19], deposition of pathogenic organisms into the duct of clinically healthy ewes did not result to clinical mastitis; thus the protective role of the intrinsic defenses of the teat was confirmed. In the sequel to that work [17], the same procedure was carried out to teats with natural or

experimentally inflicted lesions and severe clinical mastitis developed soon after challenge. We attributed this to increased colonization of damaged teat skin and to physicochemical changes hindering the normal defensive process of the mammary gland. In the current study, the bacteria entering into the teat duct after suckling provided a "natural" means of inoculation of the teat duct, which resisted invasion upwards into the parenchyma. In the teat of cows, various defense mechanisms have been described, e.g. the keratin lining in the teat duct, as well as leucocytes and non-specific antibacterial proteins in the teat cistern [20, 21]. The present findings provide field corroboration of the protective role of the healthy teats of ewes, especially after application of a challenge factor (i.e., suckling). It is noteworthy that although there were further challenges to the teats, as lambs sucked many times during the 120 min interval ("B_1" to "C" sample), there was still a reduction in infected teat ducts. This suggests that the majority of bacteria would be exterminated within the healthy teats. This defense mechanism aims at reducing bacterial populations within the teat, thus minimizing possible risk of mastitis. In fact, even natural resident flora within the teat duct may cause clinical mastitis, if teat lesions would subsequently be developed [7]. No differences were observed among ewes suckling a single lamb or twins or among periods of lactation. This is not surprising, because when suckling (i.e., in-between samples "A" and "B") every teat was exposed to challenge: in ewes with twins, each lamb sucked one teat; in ewes with a single lamb, this sucked both teats. Therefore, teats had equal opportunities for infection, and thus, no significant difference were observed. As mentioned above, sampling procedure, and therefore duration of sucking by lambs, did not appear to have an effect. Therefore, challenge opportunities, corresponding to frequency of suckling, appear to be more important for transmission of bacteria and predisposing to mastitis.

Transmission of staphylococci and of *M. haemolytica*

Staphylococci, which entered into the teat duct, might have originated from the skin of the teat or from the lips of the lambs themselves. In fact, Laukova and Marounek [13] have isolated coagulase-negative staphylococci form the upper alimentary tract of lambs, whilst Vautor and others [24] reported nasal carriage of staphylococci in sheep. Staphylococci have been traditionally considered an important mastitis pathogen in dairy ewes, likely originating from the hands of milkers. In an extensive field survey carried out in suckling ewes in Great Britain [10, 11], these bacteria were the primary causal agents of subclinical mastitis, as well as being isolated from cases of clinical mastitis. The present findings confirm that these organisms can be transferred to the mammary gland during suckling and explain their involvement in mastitis in meat-producing breeds of sheep, where no hands touch the teat. *M. haemolytica* was recovered from teat duct material only after suckling. The organism had not been isolated from those sites, seconds before initiation of suckling. This is clear evidence that the organism was transmitted from the lambs to the teat ducts. Given that Al-Sultan and Aitken [1] have reported tonsilar carriage of *M. haemolytica* in up to 100% of healthy lambs, the most probable source of the organism would be the upper respiratory tract of the lambs. This finding confirms the initial hypothesis previously presented by Scott and Jones [22] and Jones and Watkins [12]. We postulate that as the lower part of the teat comes into contact with the pharynx of the lamb [23], the organism is attached thereon, subsequently entering into the duct; perhaps the tongue of the lamb may "push" the bacteria upwards into the duct. Isolation of the organism after short (1 min) suckling activity indicates the speed by which the whole process can take place. Vilela and others [25] have documented *M. haemolytica*'s requirement for attachment on the mammary cells, in order to exhibit its pathogenicity. Perhaps the same sucking activity by the lamb can subsequently

remove from the teat bacterial cells not yet attached onto mammary epithelial cells. Although suckling ewes are frequently exposed to challenge by the organism, incidence risk of clinical mastitis associated with it, has been estimated around 5% [10]. Hence, one may suggest that differences in pathogenicity factors among the various strains are responsible for development of mastitis only in some ewes. Additionally of course, inefficiency of teat defense mechanisms would further contribute.

CONCLUDING REMARKS

The results provide clear evidence that suckling increases the risk of infection of the teat duct of ewes. Nevertheless, teats were able to withstand and minimize the infection within the next two hours. The results also show that *M. haemolytica* may be transmitted during suckling activity. Maintenance of healthy teats, e.g. free from bites or viral lesions, is important for effective defence mechanisms and consequently, for prevention of mastitis.

ACKNOWLEDGEMENTS

Funding: European Social Fund and National Resources (EPEAEK-II) ARXIMIDIS-II.

REFERENCES

[1] Al-Sultan I.I., Aitken I.D., The tonsillar carriage of Pasteurella haemolytica in lambs, J. Comp. Pathol. 95 (1984) 193–201.
[2] Barrow G.I., Feltham R.K.A., Manual for the Identification of Medical Bacteria, 3rd edn, Cambridge University Press, Cambridge, 1993.
[3] Bergonier D., Berthelot X., New advances in epizootiology and control of ewe mastitis, Liv. Prod. Sci. 79 (2003) 1–16.
[4] Bergonier D., De Cremoux R., Rupp R., Lagriffoul G., Berthelot X., Mastitis of dairy small ruminants, Vet. Res. 34 (2003) 689–716.
[5] Euzeby J.P., List of bacterial names with standing in nomenclature: a folder available on the internet, Int. J. Syst. Bacteriol. 47 (1997) 590–592. (List of Prokaryotic Names with Standing in Nomenclature. Last full update September 07, 2006. URL: http://www.bacterio.net).
[6] Ewbank R., Nursing and suckling behaviour amongst Clun-Forest ewe-lambs, Anim. Behav. 15 (1967) 251–258.
[7] Fragkou I.A., Mavrogianni V.S., Gougoulis D.A., Fthenakis G.C., The bacterial flora in the teat duct of ewes can prevent from and can cause mastitis, Vet. Res. (2007) in press.
[8] Fthenakis G.C., Prevalence and aetiology of subclinical mastitis in ewes of Southern Greece, Small Rumin. Res. 13 (1994) 293–300.
[9] Fthenakis G.C., California Mastitis Test and Whiteside Test in diagnosis of subclinical mastitis of dairy ewes, Small Rumin. Res. 16 (1995) 271–276.
[10] Jones J.E.T., Lanyon M.E., An investigation of ovine mastitis in England and Wales: preliminary results, Proc. Ann. Meet. Assoc. Vet. Teachers Res. Workers Scarborough, Great Britain, (1987) pp. (1)21–22.
[11] Jones J.E.T., Watkins G.H., Studies on mastitis in sheep at the Royal Veterinary College, Proc. Sheep. Vet. Soc. 22 (1998) 83–90.

[12] Jones J.E.T., Watkins G.H, Mastitis and contagious agalactia, in: Martin W.B., Aitken I.D. (Eds), Diseases of Sheep, 3rd edn, Blackwell, Oxford, 2000, pp. 75–80.
[13] Laukova A., Marounek M., Physiological and biochemical characteristics of staphylococci isolated from the rumen of young calves and lambs, Zentralbl. Mikrobiol. 147 (1992) 489–494.
[14] McDonald J.S., Radiographic method for anatomic study of the teat canal changes between milking periods, Am. J. Vet. Res. 36 (1975) 1241–1242.
[15] Marco Melero J.C., Mastitis in Laxta Breed Sheep: Epidemiology, Diagnosis and Control. Doctoral thesis, University of Zaragoza (1994).
[16] Mavrogianni V.S., Cripps P.J., Fthenakis G.C., Description and validation of a novel technique to study the bacterial flora of the teat duct of ewes, Small Rumin. Res. 66 (2006) 258–264.
[17] Mavrogianni V.S., Cripps P.J., Papaioannou N., Taitzoglou I., Fthenakis G.C., Teat disorders predispose ewes to clinical mastitis after challenge with Mannheimia haemolytica, Vet. Res. 37 (2006) 89–105.
[18] Mavrogianni V.S., Cripps P.J., Tzora A., Skoufos I., Fthenakis G.C., Effects of hand milking on the bacterial flora of mammary gland and teat duct of ewes, J. Dairy Res. 73 (2006) 353–356.
[19] Mavrogianni V.S., Fthenakis G.C., Brooks H., Papaioannou N., Cripps P.J., Taitzoglou I., Brellou G., Saratsis P., The effects of inoculation of Mannheimia haemolytica into the teat of lactating ewes, Vet. Res. 36 (2005) 13–25.
[20] Nickerson S.C., Resistance mechanisms of the bovine udder: new implications for mastitis control at the teat end, J. Am. Vet. Med. Assoc. 191 (1987) 1484–1488.
[21] Paulrud C.O., Basic concepts of the bovine teat canal, Vet. Res. Comm. 29 (2005) 215–245.
[22] Scott M.J., Jones J.E., The carriage of Pasteurella haemolytica in sheep and its transfer between ewes and lambs in relation to mastitis, J. Comp. Pathol. 118 (1998) 359–363.
[23] Titchen D.A., Cineradiographic studies of swallowing in the suckled lamb, Ann. Rech. Vet. 8 (1977) 483.
[24] Vautor E., Abadie G., Guibert J.M., Chevalier N., Pepin M., Nasal carriage of Staphylococcus aureus in dairy sheep, Vet. Microbiol. 106 (2005) 235–239.
[25] Vilela C.L., Fitzpatrick J., Morgan K.L., In vitro adherence and invasion of ovine mammary epithelium by Mannheimia (Pasteurella) haemolytica, Vet. J. 167 (2004) 211–213.
[26] Williams D.M., Mein G.A., Closing forces of the bovine teat canal J. Dairy Res. 54 (1987) 321–325.