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Original article

The effects of inoculation of *Mannheimia haemolytica* into the teat of lactating ewes

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Abstract – The objectives of the work described in this paper were: (i) to study the outcome of challenging ewes with *Mannheimia haemolytica*, at different sites of their teats, (ii) to compare the effects of two different isolates of the organism and (iii) to describe the features of the resulting lesions. Thirty-two ewes were used in the study and allocated into one of two groups (A or B, n = 16); they were challenged with one of two isolates of *M. haemolytica*, respectively, strain ES26L of known pathogenicity or strain VSM08L from the teat duct of a healthy ewe. Each group was further divided into four equal subgroups: the ewes in the A1/B1 subgroups were intramammarily challenged; one teat of the ewes in the A2/B2 subgroups was immersed into a broth-culture of the organisms; one teat of the ewes in the A3/B3 subgroups was inoculated 2 mm-deep, whilst one teat of the ewes in the A4/B4 subgroups was inoculated 6 mm-deep. The animals were monitored clinically, bacteriologically and cytologically before and after challenge; one animal in each subgroup was euthanised 2, 4, 7 and 11 days after challenge. All ewes in the A1/B1 subgroups developed clinical mastitis, whilst of the other animals, only one ewe in each of the A4/B4 subgroups did. Neither of the two strains used was associated with more positive bacteriological or CMT results; the A2/B2 subgroups were associated with less positive results than the A3/B3 and A4/B4 subgroups. In some ewes of the A2/B2 subgroups, mild leucocytic infiltration in the teat was evident; in the ewes of the A3/B3 subgroups, leucocytic infiltration (neutrophils, lymphocytes, plasma cells) was seen, as well as a lymphoid hyperplasia at the border between the teat duct and teat cistern; in ewes of the A4/B4 subgroups, intense subepithelial leucocytic infiltration was the salient feature. No differences were found in the severity of lesions between the two strains used or the three treatments carried out. Although strain VSM08L had been isolated from the teat duct of a healthy ewe, it caused mastitis when inoculated intramammarily; although strain ES26L is of known pathogenicity for the mammary gland, it did not cause clinical mastitis when deposited 2 mm-deep into the teat. These findings point to a protective role of the teat of ewes, which appear to limit bacterial penetration from the teat duct or cistern to the mammary gland. The lymphoid tissue, at the border between the teat duct – teat cistern, may play a significant protective role.

***Mannheimia haemolytica* / sheep / mastitis / teat / defence mechanisms**

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1. INTRODUCTION

Mannheimia haemolytica is an important causative agent of ovine mastitis in ewes sucking lambs [4, 5, 19, 22], but of lesser importance when ewes are milked [5]. Scott and Jones [34] isolated the organism from the teat skin of suckling ewes, but not from that of pregnant ewes or ewes after weaning of lambs. Subsequently, Jones and Watkins [19] suggested that the organism might originate from the nares, mouth and tonsils of lambs sucking the affected ewe. The teat orifice is the portal of entry of the bacteria, which subsequently ascend to the mammary gland through the teat [4].

Although in cows it is now well established that the teat provides a barrier to infection and the first line of defence against invading organisms [24, 29], in ewes similar studies have not been published. In previous studies of the pathogenesis of ovine mastitis [10, 11, 15, 16], the bacteria were directly inoculated into the cistern of the mammary gland. These findings provide valuable information regarding the pathogenicity of the organisms, but since the teat had been by-passed, its possible protective role was not studied.

The objectives of the work described in this paper were the following: (i) to study the outcome of challenging ewes with *M. haemolytica* at different sites of their teats, (ii) to compare the effects of two different isolates of the organism and (iii) to describe the features of the resulting lesions.

2. MATERIALS AND METHODS

2.1. Experimental design and bacterial isolates

Thirty-two lactating primiparous Karagouniko-breed ewes were used in the study. The lambs of these ewes were weaned 18 days after lambing and subsequently, the animals were hand-milked thrice daily. They were allocated into one of two groups (A or B, $n = 16$) and challenged with one of two isolates of *M. haemolytica*. Strain ES26L [10] had been isolated from a case of clinical ovine mastitis in England and is of known pathogenicity for the mammary gland of ewes; we used it by kind permission of Professor J.E.T. Jones of The Royal Veterinary College. Strain VSM08L had been isolated in three consecutive samplings, from the teat duct of a clinically healthy ewe in Greece; no abnormal clinical findings were present in the mammary gland of this animal, the California Mastitis Test (CMT) was negative and no bacteria were isolated from the mammary secretion; the organism was recovered from the tip of a fine catheter 2 mm-long, which had been inserted into the teat for sampling.

In each of the two groups, the ewes were divided into four equal subgroups ($n = 4$) and challenged at different sites of one of their mammary glands or teats (Tab. I). The experiment was carried out under a licence for experimental procedures obtained from the Greek Ministry of Agriculture.

Table I. Summary of experimental design.

Subgroup ($n = 4$)	*M. haemolytica* isolate used	Inoculation procedure
A1	ES26L	1220 c.f.u. into the gland cistern
A2	ES26L	Immersion into broth-culture (2×10^8 c.f.u.)
A3	ES26L	1220 c.f.u. 2 mm-deep into the teat
A4	ES26L	1220 c.f.u. 6 mm-deep into the teat
B1	VSM08L	1220 c.f.u. into the gland cistern
B2	VSM08L	Immersion into broth-culture (2×10^8 c.f.u.)
B3	VSM08L	1220 c.f.u. 2 mm-deep into the teat
B4	VSM08L	1220 c.f.u. 6 mm-deep into the teat

2.2. Preparation of inocu+la and the inoculation technique

For inoculation, each isolate was grown on Columbia blood agar and checked for purity; then it was inoculated into Soy-broth (BioMerieux) and incubated aerobically at 37 °C for 5 h.

For ewes in the A2/B2 subgroups, the whole broth culture was used. The ewes were completely milked out; three hours later, the lower 3 cm of the teat were immersed into 40 mL of the broth for 60 s; after a 30 min break, they were re-immersed into the same broth culture. The procedure was repeated on the following day. The other teat of each ewe was immersed into 40 mL of sterile phosphate-buffer-saline (PBS), pH 7.3 using the same procedure.

For ewes in the other subgroups, serial dilutions of the broth culture into PBS were carried out; finally, 0.2 mL of the desired dilution was withdrawn with a syringe. The inoculum contained 1220 c.f.u., as estimated by the method of Miles and Misra [23]. To ensure sterile conditions, on the day before inoculation the hairs of the teats were clipped using fine scissors and the skin of the udder and teats was scrubbed using chlorhexidine; then on the day of inoculation iodine povidone solution was used for animals of the A1/B1, A3/B3 and A4/B4 subgroups. The ewes in the A1/B1 subgroups were inoculated directly into the mammary gland cistern, as described before [11, 16]. The ewes in the A3/B3 and A4/B4 subgroups were challenged as follows; a sterile plastic fine catheter (Abbocath, Abbot) 20 G, 2 mm- or 6 mm-long respectively, was inserted into the teat; the syringe was attached to the catheter and the bacterial suspension was deposited inside the teat (Tab. I). The same technique was used to inject 0.2 mL of PBS into the other mammary gland or at the respective site of the other teat of each ewe and to be used as a control.

2.3. Pre- and post-inoculation examinations

Immediately after lambing and every two days thereafter, as well as on the day of inoculation (20th day after lambing), a thorough clinical examination was carried out in the ewes. Special attention was paid to their mammary glands, which were examined as described before [13], and teats. Each teat was held between the thumb and the index finger of the examiner (VSM) and palpated throughout its length; its shape, size and consistency were evaluated. The teats of the same ewe were compared to each other. Subsequently, the skin around the teat orifice and in the lower lateral part (approx. 1 cm) of the teat was swabbed by means of a sterile cotton-swab moistened in Soy-broth. A sterile plastic fine 2 mm-long catheter was inserted into the teat and moved from the left to right, in order to sample the mucosa. Then, mammary secretion samples were obtained. The first two squirts of secretion were discarded and then, 10 to 15 mL of secretion were carefully collected into a sterile container.

All samples were cultured onto Columbia blood agar; the media were incubated aerobically at 37 °C for up to 72 h. The CMT was carried out in secretion samples, as described before [14]; secretion films were made and stained by the Giemsa method.

After challenge, detailed examinations of the mammary glands and teats were carried out daily. In ewes of the A2/B2 subgroups, sterile cotton-swabs moistened in Soy-broth were used to sample the skin around the teat orifice and the lower lateral part (approx. 1 cm) of the teat before disinfection. In one of the four ewes of each of the A2/B2, A3/B3 and A4/B4 subgroups, a sterile plastic fine 2 mm-long catheter was inserted into the teat to sample the mucosa. Mammary secretion samples were collected from all experimental animals. All samples were cultured onto Columbia blood agar; the media were incubated aerobically at 37 °C for up to 72 h. The CMT was carried out in all secretion samples. Secretion films

were made and stained by the Giemsa method; the percentage of leucocyte subpopulations was determined by counting at least 200 cells therein and determining their type.

One of the ewes in each of the eight subgroups was euthanised on each of D2, D4, D7 and D11. Dissection of the mammary glands and the teats started immediately and was carried out using the aseptic technique. The skin of the teats and the subcutaneous tissues were incised with a sterile blade; initially the mucosa of the teat cistern (*sinus papillaris*) was exposed and subsequently the teat duct (*ductus papillaris*) was incised and its mucosa was exposed. A new blade was used for scraping the mucosa of the teat cistern, whilst another one was used to scrape the mucosa of the teat duct. In ewes of the A3/B3 and A4/B4 subgroups, an electronic cutimeter was used to measure 2 mm or 6 mm respectively, from the teat orifice, in order to determine the precise site of the teat, where the inoculum had been deposited. Then, the mammary glands were dissected and samples were obtained for bacteriological and histological examination, as described before [11]. All tissue samples obtained were plated onto Columbia blood agar; the media were incubated aerobically at 37 °C for up to 72 h. Throughout this study, all isolated bacteria were identified using conventional techniques [3].

Longitudinal sections, involving all the structures of the teat, were carried out for histopathological examination. Tissue samples were fixed in 10% neutral-buffered formalin and embedded in paraffin wax. Haematoxylin and eosin (HE) standard staining procedures were performed for histopathological studies.

2.4. Data management and analysis

The number of bacteriologically positive and CMT positive results obtained by each of the two strains used, as well as by each of the three different procedures of challenging the teats, were compared between them.

Table II. Description of scores given for pathological findings in the teats of ewes inoculated with *M. haemolytica*.

Score	Description
Macroscopic pathological findings	
0	Normal
1	Presence of folds on the mucosa of the teat
2	Hyperaemia of the mucosa of the teat
3	Thickening of the mucosa of the teat, with increased number of folds and presence of petechiae
4	Extreme thickening of the mucosa of the teat, with loss of the separation of the compartments of the teat
Histopathological findings	
0	Normal
1	Presence of a few, scattered leucocytes
2	Presence of increased numbers of leucocytes clustered under the epithelium of the teat
3	Presence of high numbers of leucocytes evenly distributed under the epithelium of the teat
4	Presence of high numbers of leucocytes, plus hyperplasia of lymphoid tissue in the teat duct – teat cistern border

A scoring system was devised and numerical values were assigned for the pathological findings in the teats of the experimental animals. A separate score (0–4 scale) was given for macroscopic and for histological findings; these were then added to a 0–8 scale to produce a pathology score for findings in the teats. The system is detailed in Table II.

The number of bacteriologically positive results from teat scrapings obtained by each of the two strains used, as well as by each of the three different procedures of challenging the teats, was compared between them. The sum of all teat pathology scores for animals challenged with strain ES26L

was compared to that of animals challenged with isolate VSM08L. The Wilcoxon 1-sample test for the median was employed to test for differences between each pathology score and zero, as well as between differences in medians among macroscopic, histological and total pathology scores. The Friedman non-parametric test was used to test for differences in pathology score medians among the three different challenging procedures, as well as among the days post-infection when the ewes were euthanised. Statistical significance was defined as $P < 0.05$.

3. RESULTS

3.1. Pre-inoculation examinations

The mammary glands and the teats of all ewes were found to be clinically healthy during the period from lambing to inoculation. The teats were soft with no external abnormalities. A variety of bacteria was isolated from all teat skin swabs; the majority of the organisms was coagulase-negative *Staphylococcus* spp., but other bacteria (e.g. *Bacillus* spp., *Acinetobacter* spp.) were also isolated. *M. haemolytica* was also recovered sporadically after the 12th day after lambing (1 A1 ewe, 2 B2 ewes, 2 A3 ewes, 1 A4 ewe, 1 B4 ewe). No bacteria were isolated from any catheter or milk samples obtained. The CMT was always negative; in Giemsa-stained secretion films, no leucocytes were observed.

3.2. Clinical, bacteriological and cytological findings

All the ewes in the A1/B1 subgroups developed clinical mastitis within 24 h after challenge. There were changes in the mammary secretion (serous or sero-haemorrhagic appearance, with flakes) and the inoculated glands (enlarged, hot, with a red-coloured skin). *M. haemolytica* was consistently isolated from the mammary secretion. The CMT increased (> "2") and leucocytes were present in Giemsa-stained secretion films.

None of the ewes in the A2/B2 subgroups developed clinical mastitis; however, three ewes (two of those challenged with ES26L and one of those challenged with VSM08L) developed subclinical mastitis. *M. haemolytica* was isolated in pure or mixed culture from the teat skin swabs until D8. It was also isolated in pure culture from catheter samples from D1 until D11 and from secretion samples from D7 until D11. The CMT increased (> "1") already on D1 in some ewes and by D4 in most ewes. Leucocytes were seen in Giemsa-stained secretion films; up to the 4th–5th day after challenge, the great majority (≥ 95%) of leucocytes consisted of neutrophils with a few macrophages and lymphocytes also present; subsequently, the percentage of neutrophils decreased (20–70%), whilst those of macrophages and lymphocytes increased (20–40% and 20–50%, respectively).

None of the ewes in the A3/B3 subgroups developed clinical mastitis. *M. haemolytica* was isolated in pure culture from catheter and secretion samples from D1 until D11. The CMT increased (> "1") already on D1 in most ewes. Leucocytes were seen in Giemsa-stained secretion films; up to the 2nd day after challenge, the great majority (≥ 85%) of leucocytes consisted of neutrophils with a few macrophages and lymphocytes (5–10%) also present; subsequently, the percentage of neutrophils decreased (40–60%), whilst that of macrophages and lymphocytes increased (5–30% and 10–40%, respectively).

Two of the ewes in the A4/B4 subgroups (one in either subgroup) developed clinical mastitis; the secretion was thick and contained flakes, the teats were hot and reddened and the mammary glands were enlarged. *M. haemolytica* was isolated in pure culture from catheter and secretion samples from D1 until D11. The CMT increased (> "1") already on D1 in all ewes. Leucocytes were seen in Giemsa-stained

Table III. Sequential *M. haemolytica* isolation (bacteriological findings) and results of CMT in samples from ewes with teats inoculated with the organism.

	Subgroup																	
	D1[a]			D2[a]			D3[a]			D4[a]			D5[a]			D6[a]		
	2[b]	3[b]	4[b]	2	3	4	2	3	4	2	3	4	2	3	4	2	3	4
Bacterial isolation																		
Teat skin	8/8[c]	–	–	8/8	–	–	6/6	–	–	6[d]/6	–	–	2[d]/4	–	–	1[d]/4	–	–
Teat duct	2/2	2/2	2/2	2/2	2/2	2/2	1/2	2/2	2/2	2/2	1/2	2/2	2/2	1/2	2/2	1/2	0/2	2/2
Secretion	0/8	3/8	4/8	0/8	5/8	5/8	0/6	4/6	5/6	0/6	5/6	4/6	0/4	4/4	3/4	0/4	3/4	2/4
CMT results																		
Positive*	2/8	6/8	8/8	3/8	8/8	7/8	3/6	5/6	5/6	4/6	5/6	5/6	3/4	4/4	1/4	3/4	4/4	1/4

	D7[a]			D8[a]			D9[a]			D10[a]			D11[a]		
	2[b]	3[b]	4[b]	2	3	4	2	3	4	2	3	4	2	3	4
Bacterial isolation															
Teat skin	1[d]/4	–	–	1[d]/2	–	–	0[d]/2	–	–	0[d]/2	–	–	0[d]/2	–	–
Teat duct	2/2	1/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	1/2	2/2	1/2	2/2	2/2	2/2
Secretion	2/4	3/4	3/4	1/2	2/2	2/2	2/2	2/2	2/2	1/2	2/2	2/2	1/2	2/2	2/2
CMT results															
Positive	3/4	4/4	3/4	1/2	1/2	2/2	2/2	2/2	0/2	0/2	1/2	2/2	1/2	1/2	2/2

[a] D1, D2, etc. = days after challenge.
[b] 2 = A2 and B2 subgroups, 3 = A3 and B3 subgroups, 4 = A4 and B4 subgroups.
[c] n/m = positive results out of total animals sampled.
[d] Bacteria other than *M. haemolytica*, isolated as well.

secretion films; up to the 2nd day after challenge, the great majority (≥ 80%) of leucocytes consisted of neutrophils with a few macrophages and lymphocytes (5–15%) also present; subsequently, the percentage of neutrophils decreased (10–70%), whilst that of macrophages and lymphocytes increased (0–20% and 10–60%, respectively).

Detailed bacteriological and CMT results in samples from animals in the A2/B2, A3/B3 and A4/B4 subgroups are in Table III. Neither of the two strains used (ES26L or VSM08L) was associated with more positive results (positive bacteriological – CMT results: 64%–78%, 64%–60%, for the two strains respectively). The A2/B2 subgroups were associated with less positive results than the A3/B3 and A4/B4 subgroups (positive bacteriological – CMT results: 37%–52%, 74%–85%, 79%–75%, for the three procedures respectively).

No clinical signs were observed in any of the control glands and teats, injected with or immersed into PBS. No bacteria were isolated from any catheter of secretion sample obtained from these animals. A variety of bacteria was isolated from the teat skin swabs; the majority of the organisms was again coagulase-negative *Staphylococcus* spp., but other bacteria (e.g. *Staphylococcus aureus*, *Bacillus* spp., *Acinetobacter* spp.) were also isolated; no *M. haemolytica* was recovered. The CMT was always negative; in Giemsa-stained secretion films, no leucocytes were observed.

3.3. Pathological findings

In ewes of the A1/B1 subgroups, gross and histological lesions were those of *M. haemolytica* mastitis. The glands were markedly swollen and the affected parenchyma was reddened; the red areas were multifocal and sharply demarcated from adjacent tissue; there was marked distension of veins and an occasional presence of firm, grey clots adhering to the intima. The ducts were plugged by threads of fibrin and clots of milk; the milk ducts and cistern were filled with brown masses of exudates with threads of fibrin and debris. There were widespread neutrophilic infiltration, intramammary haemorrhages, lysis of neutrophils, destruction of epithelial cells and alveoli. No abnormal features were seen in the teats of these ewes.

In the ewes of the A2/B2 subgroups euthanised on D2, D4 or D7, the teat duct appeared normal with an apparently smooth internal lining, clearly distinguished from the teat cistern; in the teat of ewes euthanised on D11, the salient feature was the thickening of the mucosa of the teat duct. Histologically, there was leucocytic infiltration prominent at the end of the teat duct in the ewes euthanised on D2, at the border between the teat duct and teat cistern in the ewes euthanised on D4 (neutrophils, lymphocytes, plasma cells), and at the teat cistern in the ewes euthanised on D7 or D11 (lymphocytes, plasma cells); all leucocytes were observed under the epithelium of the teat. No gross pathological findings were evident in the mammary parenchyma or the supra-mammary lymph nodes; histologically, leucocytic infiltration was evident in ewes euthanised on D7 or D11, whilst no lesions were seen in ewes euthanised on D2 or D4. The organism was recovered from the teat duct and the teat cistern, but not from the mammary parenchyma or the supra-mammary lymph nodes of the inoculated ewes (Tab. IV).

The measurement of the length of the internal teat structures after dissection of the teats of ewes of the A3/B3 subgroups showed that the inoculum had always been deposited within the teat duct. Macroscopically, the teat duct of ewes euthanised on D2 or D4 appeared normal with an apparently smooth internal lining; the teat duct and the teat cistern were clearly distinguished as two different anatomical structures. In the teats of the ewes euthanised on D7, the internal lining of the teat appeared rough, whilst petechiae were seen in the mucosa of the teat cistern. In the teats of the ewes euthanised on D11, thickening of the mucosa of the teat duct resulting in partial stenosis was seen. Histologically, there was leucocytic infiltration prominent at the border between the teat duct and teat cistern in the ewes euthanised on D2 (neutrophils, lymphocytes, plasma cells) and at the teat cistern in the ewes euthanised on D4, D7 or D11 (lymphocytes, plasma cells); the leucocytes were observed under the epithelium of the teat. An area of hyperplastic lymphoid tissue consisting of lymphocytes and plasma cells was observed at the border between the teat duct and teat cistern (Fig. 1). No gross pathological findings were evident in the mammary parenchyma or the supra-mammary lymph nodes; no histological lesions were seen in ewes euthanised on D2 or D4; in ewes euthanised on D7 or D11, leucocytic infiltration (neutrophils and lymphocytes), lysis of neutrophils, extravasation and destruction of epithelial cells were evident. The organism was recovered from the teat duct, the teat cistern and the mammary parenchyma, but not from the supra-mammary lymph nodes of the inoculated ewes (Tab. IV).

The measurement of the length of the internal teat structures after dissection of the teats of ewes of the A4/B4 subgroups showed that the inoculum had been deposited within the teat cistern. Macroscopically, the mucosa of the teat duct and the teat cistern of ewes euthanised on D2 and D4 appeared rough and no clear distinction between the teat duct and the teat cistern was evident. In the teat of the ewes euthanised on D7, the internal lining of the teat appeared rough and petechiae were seen in

Table IV. Post-mortem isolation of *M. haemolytica* from ewes whose teats were inoculated with the organism.

Site where isolated	Day when ewe was euthanised											
	D2[a]			D4[a]			D7[a]			D11[a]		
	2[b]	3[b]	4[b]	2	3	4	2	3	4	2	3	4
Teat duct	1/2	2/2	2/2	2/2	2/2	2/2	1/2	2/2	2/2	2/2	2/2	2/2
Teat cistern	0/2	1/2	2/2	1/2	2/2	1/2	1/2	1/2	1/2	0/2	2/2	2/2
Mammary parenchyma	0/2	1/2	1/2	0/2	1/2	0/2	0/2	0/2	0/2	0/2	2/2	1/2

[a] D2, D4, etc. = days after challenge.
[b] 2 = A2 and B2 subgroups, 3 = A3 and B3 subgroups, 4 = A4 and B4 subgroups.

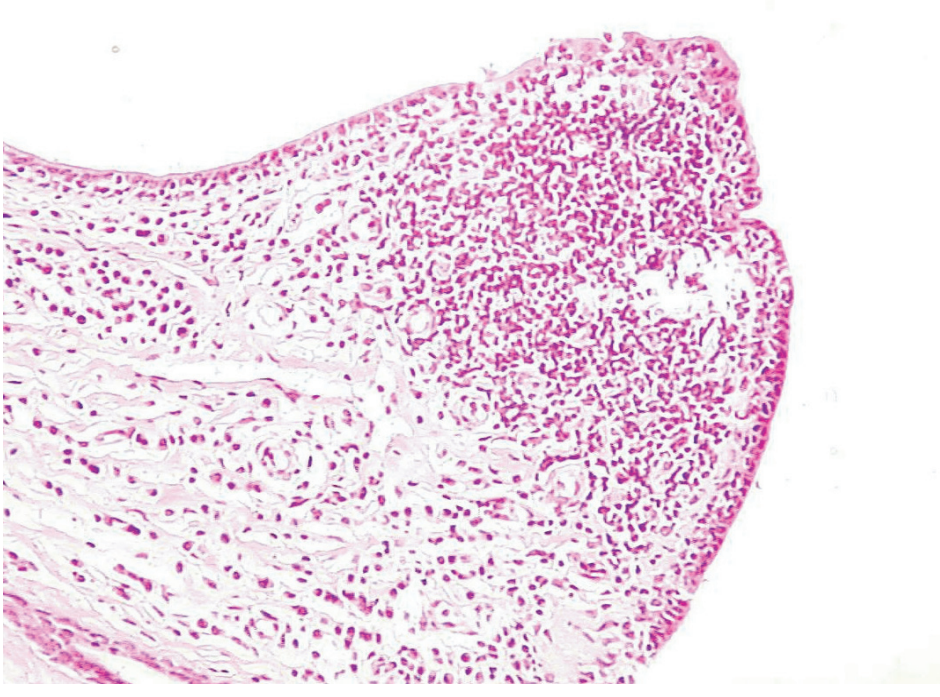

Figure 1. Hyperplastic lymphoid tissue consisting of lymphocytes and plasma cells at the border between the teat duct and teat cistern (low magnification 10× objective, photograph taken on a Zeiss photomicroscope III) (ewe challenged into the teat duct with isolate VSM08L and euthanised on D11).

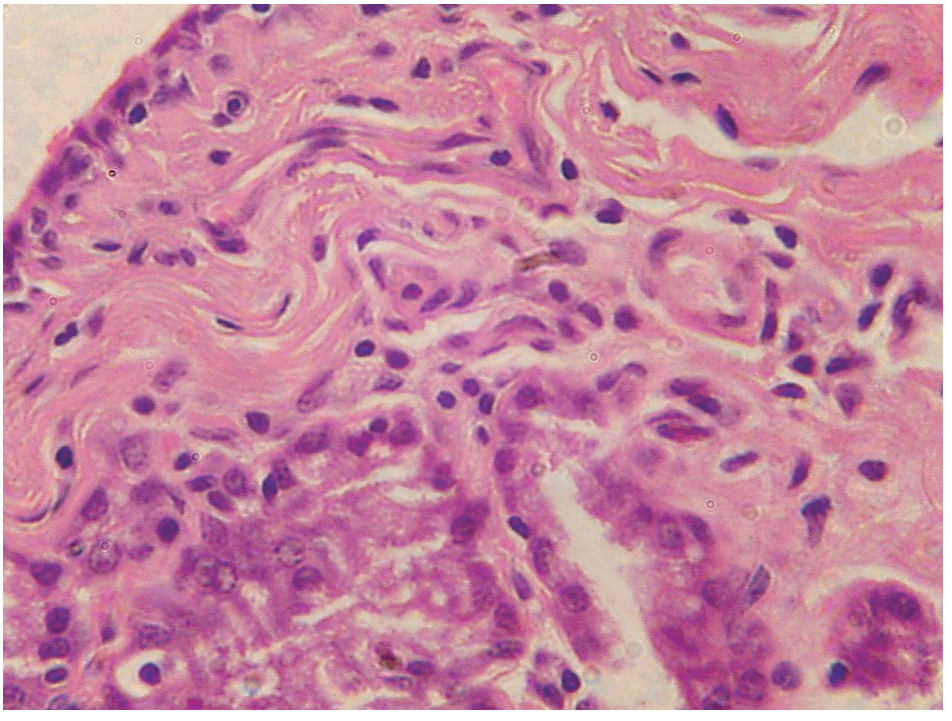

Figure 2. Subepithelial leucocytic infiltration in the teat cistern (lymphocytes, plasma cells) (medium magnification 40× objective, photograph taken on a Zeiss photomicroscope III) (ewe challenged into the teat cistern with isolate ES26L and euthanised on D11).

the mucosa of the teat cistern. In the teat of the ewes euthanised on D11, thickening of the mucosa of the teat duct and cistern was seen. Histologically, there was leucocytic infiltration in the teat cistern (neutrophils, lymphocytes, plasma cells); the leucocytes were observed under the epithelium of the teat (Fig. 2). No area of hyperplastic lymphoid tissue was observed at the border between the teat duct and teat cistern. No gross pathological findings were evident in the mammary parenchyma or the supramammary lymph nodes, but massive leucocytic infiltration was seen histologically; furthermore, lysis of neutrophils, extravasation and destruction of epithelial cells and alveoli were evident. The organism was recovered from the teat duct, the teat cistern and the mammary parenchyma, but not from the supra-mammary lymph nodes of the inoculated ewes (Tab. IV).

No macroscopic or histological pathological findings were observed in the control teats and mammary glands of the experimental animals. All pathology scores were 0. *M. haemolytica* was not isolated from the control teats and mammary glands.

Neither of the two strains used (ES26L or VSM08L) was associated with more positive results (positive bacteriological results: 56%, 61%, for the two strains respectively). The A2/B2 subgroups were associated with less positive results than the A3/B3 and A4/B4 subgroups (positive bacteriological results: 33%, 75%, 67%, for the three procedures respectively).

Details of the pathology scores are in Table V. No clear pattern of differences

Table V. Total pathology scores for findings in the teats of ewes challenged with *M. haemolytica.*

Subgroup	Day when ewe was euthanised			
	D2[a]	D4[a]	D7[a]	D11[a]
A2	4	1	5	7
A3	4	1	7	6
A4	6	3	4	5
B2	3	4	3	6
B3	5	4	6	8
B4	2	3	5	5

[a] D2, D4, etc. = days after challenge.

between the scores obtained from teats inoculated with either isolate was evident; the sum of all pathology scores for animals challenged with ES26L (= 53) was close to that for experimental animals challenged with VSM08L (= 54). A tendency for scores to increase with day post-infection was evident, but this was not statistically corroborated. However, scores for each day and treatment were statistically significant from zero, i.e. the control teats ($P = 0.036$). Furthermore, no significant differences were found either among the three treatments, i.e. the A2/B3, A3/B3, A4/B4 subgroups ($P = 0.180$), or among the macroscopic, histological and total pathology score ($P = 0.058$).

4. DISCUSSION

In previous experimental studies of ovine mastitis associated with *M. haemolytica*, the bacteria were deposited directly into the gland cistern; that way, the teat had been by-passed. In our work, we inoculated two *M. haemolytica* strains around or into the teat of ewes; the results are interesting for the assessment of the protective role of the teat of ewes.

Animals in the A1/B1 subgroups were used as positive controls; the organisms were inoculated directly into the cistern of their mammary gland and clinical mastitis was consistently induced. For isolate ES26L, this confirmed its pathogenicity for the mammary gland [10, 11]; for VSM08L, it showed that the strain was pathogenic for the mammary gland.

The latter strain had been isolated from the teat duct of a healthy ewe, but was found to be pathogenic for the mammary gland when inoculated directly into the gland cistern. One may thus suggest that in the ewe, from which this strain had originally been isolated, there was an equilibrium between host defences and bacterial virulence factors; this was changed in the experimental animals, where we inoculated the organism directly into the gland cistern, resulting in mastitis. This finding suggests that the healthy teat of the original animal provided protection for the mammary gland. It also indicates that teats harbouring bacteria can be a source of infection for the mammary gland; any impediment of the defence mechanisms may shift the balance and allow the bacteria to multiply, invade the mammary gland and cause mastitis.

The survival of the organism and its continuous isolation from the skin of teats after immersion (A2/B2 subgroups) underlines the risks for infection of the mammary glands of ewes. The organism colonizes the teat skin of ewes during the suckling period, as found in the study of Scott and Jones [34]. Lambs may suck up to 32 times daily [2] and thus, the risks of infection are perennial. Sucking itself could contribute towards the ascent of the organisms, which may possibly be "pushed" into the mammary gland. On the contrary, one may suggest that sucking would contribute to removing the invading bacteria; in an attempt to simulate these conditions, ewes were milked thrice a day and their mammary glands were completely emptied. Nevertheless, the bacteria successfully ascended to the mammary parenchyma, as established by the results of bacteriological and cytological tests.

Clinical mastitis was induced in two ewes, in which bacteria were deposited into

their teat cistern, but in none of the ewes of the other experimental subgroups. Furthermore, although El-Masannat [10] was able to induce clinical mastitis after intramammary inoculation of as few as 7 c.f.u. of isolate ES26L, no clinical mastitis was induced in the ewes of the A3/B3 subgroups, although a hundred-seventy fold higher dose was deposited into their teat duct.

These findings further point out to a protective role of the teat of ewes. Bacteria deposited into the teat duct were able to ascend to the mammary gland, but did not cause clinical mastitis. In cows, the teat provides important protection for the mammary gland [29]. The keratin of the teat duct inhibits proximal progression of bacteria [6, 17, 33], subsequent cellular and humoral defence mechanisms occur in the teat cistern, e.g. leucocytes and non-specific antibacterial proteins [1, 8, 9, 28, 30–32].

Although our animals had been sampled and no bacteria were isolated from the teat duct before challenge, under field conditions one may also postulate that the bacterial flora residing in the teat duct of healthy ewes [16] acts competitively to the invading bacteria, slowing down their growth and rendering them more susceptible to the defence mechanisms of the mammary gland. In fact, Linde [20] found that in cases of the presence of coagulase-negative staphylococci inside the mammary gland of cows, it is difficult to experimentally induce mastitis. These organisms are the most frequently isolated bacteria from the teats of ewes [16], and therefore, one may postulate that they may also afford a protection against invading bacteria at the teat duct.

Similar studies have not been previously reported in ewes. Furthermore, knowledge from cows cannot be applied directly to ewes. Husbandry systems applied in sheep expose the teats to stress factors differing to those in cows' teats. In mutton breeds, ewes suckle their lambs for three to four months, whilst in dairy breeds, ewes are initially sucked and then -in the majority of cases- hand-milked.

One interesting question is why the bacteria, although they reached the mammary gland, did not cause clinical mastitis. We believe that the neutrophilic response elicited after bacterial deposition into the teat (Colditz and Presson [7] found that in cows' leucocytes invaded the teat within two hours after challenge) prepared the mammary gland to counter-act the invading bacteria when these reached the mammary parenchyma. When inoculation had been carried out directly into the gland cistern, neutrophils were found to enter into the mammary gland 12 h after challenge [10, 15], by which time multiplication of bacteria had taken place and the bacterial numbers involved could not be destroyed by the neutrophils. One may also suggest that some bacteria would die within the teat duct and teat cistern during their ascent and ultimately, smaller numbers of bacteria entered the mammary parenchyma.

In a previous paper [21], we described that a pathogenic strain of *Staphylococcus chromogenes* causes mild transient clinical mastitis four days after its deposition into the teat duct of ewes; however, when it had been inoculated directly into the gland cistern, it caused severe, fatal clinical mastitis within 24 h [16]. Furthermore, in a field study and using ultrasonography to measure the dimensions of teat ducts of ewes, Franz et al. [12], found that in ewes with longer teat ducts, mastitis is less prevalent, whilst in ewes with wider teat ducts mastitis is more frequent. These findings lend further support to our hypothesis, confirming that the teat duct appears to play an important protective role in the mammary gland of ewes.

All the above evidence, points to a protective role of the normal teat of ewes. The lymphoid tissue observed at the border between the teat duct-teat cistern, may play a protective role, as in histological sections from teats inoculated 2 mm-deep, we recorded that it was hyperplastic and producing lymphocytes. No such findings have been previously reported in ewes. In cows

the lymphoepithelial structure previously termed "Furstenberg's rosette", is considered to be important for the elimination of invading microorganisms [18]. Nickerson and Pankey [25, 26] and Nickerson et al. [27] found evidence of an immunological potential in teat tissues around that area, likely as a consequence of the presence of germinal centres and accumulation of plasma cells. This appears to be an interesting area for further research and points out that anatomical differences between cows and ewes further emphasise the point raised above, that evidence from cows cannot be applied directly to ewes.

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