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# Bacteriological and epidemiological findings during examination of the uterine content of ewes with retention of fetal membranes

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**Abstract**

We included 92 pairs of ewes with or without retention of fetal membranes in a cohort study of 25 flocks in Southern Greece. We obtained two uterine content samples under aseptic conditions, by introducing a swab into the uterus of these ewes, on the 2nd–4th and the 5th–9th day after lambing. We used conventional bacteriological techniques to isolate and identify bacteria and to carry out antimicrobial agents susceptibility testing. The prevalence of bacterial intrauterine contamination among ewes with retention was 24% on the first and 46% on the second sampling ($P < 0.0001$) and that among ewes without retention was 8 and 2% ($P > 0.05$), respectively. Clinical signs accompanying the retention of fetal membranes were more frequently observed among ewes with intrauterine contamination than among those without ($P = 0.0007$). The odds of an ewe having an intrauterine contamination increased multiplicatively by 1.06 when the median duration of retention in the flock increased by 6 h. The principal bacteria isolated from the ewes with retention were *Arcanobacterium pyogenes* and *Escherichia coli*; 21% of 73 isolates tested were found resistant to at least one antimicrobial agent. 

*Keywords:* Sheep reproduction; Fetal membranes; Infection; Metritis; *Arcanobacterium pyogenes*

## 1. Introduction

Postpartum intrauterine contamination has been studied extensively in cows. The genital tract is particularly susceptible to infections immediately after calving, but it is usually able to overcome the nonspecific bacterial contamination [1]. In an early study, ~93% of dairy

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cattle were found to have a varying degree of bacterial contamination of their uterus during the early postpartum period, but this decreased to 9% 46–60 days after calving [2]. This is probably related to the large amount of neutrophils, physiologically found in the uterus during the early puerperium [3,4]. Various risk factors have been associated with postpartum uterine infection [5]. Among them, retention of fetal membranes particularly predisposes cows to the disorder [6]; in fact, the incidence of metritis in cows with retention has been reported to vary from 37 to 90% [5].

To our knowledge, similar studies do not exist in ewes. Retention of fetal membranes in ewes, with the small incidence risk of 1.25% [7], has not been studied extensively; furthermore, no bacteriological investigations of cases of retention have been reported.

This study presents the results of a bacteriological investigation of the uterine content of ewes with retention of fetal membranes. Our objectives were to describe the prevalence and the identity of bacteria in the uterus of ewes with retention, to investigate their pattern of susceptibility to antimicrobial agents, and to study the possible association of intrauterine contamination with some clinical parameters of the disorder. We carried out the investigation as part of a larger work related to retention of fetal membranes in ewes, carried out in 28 flocks in Southern Greece.

## 2. Materials and methods

### *2.1. Design of the investigation*

We conducted a cohort study in 25 flocks of dairy ewes in Southern Greece. The exposure factor was the retention of fetal membranes, which was defined as the failure to expel the placenta(e) within 6 h after lambing the last lamb. To account for the likely flock-effect, we matched each exposed ewe to a nonexposed one [8]. We achieved this by selecting for the control group the first ewe that lambed after the exposed ewe (within the same flock) and expelled her placenta(e) within 6 h after lambing the last lamb. In total, we included 92 pairs of exposed and nonexposed ewes in the study (details on management of the flocks reported by Fthenakis et al. [7]).

### *2.2. Sampling technique and bacteriological examination*

We carried out an initial sampling for bacteriological examination in all studied ewes ($92 + 92$) on the 2nd–4th day after lambing. We obtained a second sample 2–5 days later, i.e., on the 5th–9th day after lambing.

We restrained the ewe and lifted her tail; then, we washed the perineal and vulval areas with a povidone–iodine solution (Mundipharma SA, St. Alban-Rheinweg, Switzerland) and warm water. We parted the vulval lips and we advanced a guarded culture instrument (Medical Wire & Equipment Co., Corsham, UK) into the uterus. We pushed the swab of the instrument out of its protective sheath, moved it into the uterus and submerged it into its content. Then, we retracted it into its cover and withdrew it from the vagina. We maintained the swabs in a transportation medium, transferred them to the laboratory within an isothermic box; cultured them within 24 h after collection.

We plated each swab onto Columbia 5% sheep blood agar (Oxoid Ltd., Basingstoke, UK) plates, incubated the plates aerobically and anaerobically at 37 °C and examined them after 24 h. We achieved anaerobic conditions by using a Gas-Pak system (BBL Microbiology Systems Inc., Cockeysville, MD, USA). If nothing had grown, we reincubated the plates up to a further 48 h and re-examined them. If bacteria grew, we described the characteristics of the colonies and recorded their growth. We identified them according to the methods of Barrow and Feltham [9] and we speciated the staphylococcal and streptococcal isolates by using the 'API-Staph IDENT SYSTEM' and the 'API-Strep IDENT SYSTEM' (Bio-Merieux, Lyon, France), respectively.

### 2.3. Antimicrobial agents susceptibility testing

We performed antimicrobial agents susceptibility testing on 73 different bacterial isolates (24 *Arcanobacterium pyogenes* (*A. pyogenes*), 24 *Escherichia coli* (*E. coli*), 16 *Staphylococcus* sp., 9 *Streptococcus* sp.) from uterine content samples obtained on the first or second sampling from ewes with or without retention.

We used disks (Difco Laboratories, Detroit, MI, USA) containing ampicillin (10 μg), clindamycin (2 μg), enrofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), neomycin (30 μg), penicillin-G (10 i.u.) and tetracycline (30 μg); these antimicrobial agents are the ones commonly used in Greece for postparturient administration into ewes. We also used one disk without antibiotic on each Petri dish as negative control indicator, and *Staphylococcus aureus* (*Staph. aureus*) strain NCTC6571 (National Collection of Type Cultures, London, UK) as the control organism.

We prepared test and control organisms identically and examined them simultaneously. We suspended colonies from a blood–agar plate in 2 ml sterile saline to a density approximately equal to MacFarlands Opacity Standard No. 1. We immersed a dry cotton wool swab into the suspension and drained excess liquid by expressing it against the inside of the bottle. We inoculated the bacterial suspension onto Iso-SensiTest agar (Oxoid) with the swab in such a way that the whole surface of the agar was covered. Then, we applied the disks.

We incubated the plates aerobically (*E. coli*, staphylococci) or anaerobically (*A. pyogenes*, streptococci) for 18 h at 37 °C and subsequently recorded the results as susceptible, moderately susceptible, intermediate or resistant by measuring the inhibition zone diameter [10] according to the interpretive standards of Difco [11] and by comparing the inhibition zone sizes to those of the control organism.

### 2.4. Calculations and statistical analysis

We defined the duration of retention of fetal membranes as the length of time during which the fetal membranes had not been expelled. We classified each ewe on one of the following time intervals, according to the duration of retention: 6–12 h, >12–18 h, >18–24 h, >1–2 days, >2–3 days, >3–4 days, >4–6 days, >6–8 days, >8–10 days and >10–14 days (for calculations, we assumed that expulsion occurred at half-time of each of the above intervals, i.e., 9, 15, 21, 36, 60, 84, 120, 168, 216 and 288 h after lambing, respectively).

We classified each ewe with retention into a clinical score from 1 to 5 as follows—Score 1: retention of fetal membranes for 6–12 h; Score 2: retention of fetal membranes

for >12 h with no clinical symptoms accompanying it; Secore 3: retention of fetal membranes for >12 h with mild genital symptoms accompanying it; Score 4: retention of fetal membranes for >12 h with moderate systemic or genital symptoms accompanying it; Score 5: retention of fetal membranes for >12 h with severe systemic or genital symptoms accompanying it [7].

We calculated the prevalence of intrauterine contamination from the formula: $IP_t = N/A$, where $N$ is the number of animals with the event of interest and $A$ is the number of animals at risk at that point in time [12].

We compared the prevalence of intrauterine contamination in ewes with retention to that in ewes without retention by using Pearson's $\chi^2$ test in StatXact-4 for Windows (CYTEL Software Corporation, Cambridge, MA, USA). We applied the same test in order to examine the association between intrauterine contamination and the existence of clinical signs. Within ewes with or without retention, we compared the prevalence of intrauterine contamination on the first sampling to that on the second using McNemar's $\chi^2$ test for symmetry in StatXact-4 (CYTEL Software Corporation). We also compared the frequency of isolation of *A. pyogenes* or *E. coli* (the two most frequently isolated bacterial species) from ewes with retention in the first sampling, to that in the second sampling using the same test as above.

We compared the frequency of each clinical sign accompanying retention among ewes, from the uterine sample of which bacteria were isolated, to that among ewes, from the uterine sample of which no bacteria were isolated, using Pearson's $\chi^2$ test in StatXact (CYTEL Software Corporation). To adjust for multiple comparisons, we set the $P$ value for significance to $P < 0.05$ (Bonferonni adjustment).

We examined the within-flock prevalence of intrauterine contamination in the first or the second sampling for association with the median duration of retention and the median clinical score of ewes with retention, in each flock, using logistic regression models, fitted in SAS Proc Logistic, rel. 6.12 (Statistical Analysis Systems, Cary, NC, USA). In these models, the outcome was in the form of binomial proportions, for which the numerator was the number of bacteriologically positive ewes with retention and the denominator was the number of ewes with retention in each flock. We evaluated the need for adjustment for overdispersion in the data by examination of the mean deviances [13]. From all these analyses, we excluded the three flocks with only one ewe with retention.

We used the 5% significance level for all statistical evaluations.

## 3. Results

### 3.1. Prevalence of intrauterine contamination and identity of bacterial isolates

The prevalence of bacterial intrauterine contamination among ewes with retention was 24% on the first and 46% on the second sampling. The prevalence among ewes without retention was 8 and 2%, respectively. The difference in the prevalence rate between the ewes with or without retention was statistically significant, both on the first ($P = 0.004$) and on the second sampling ($P < 0.0001$). The increase of the prevalence of intrauterine contamination from the first to the second sampling, among ewes with retention, was statistically significant ($P < 0.0001$). We found no statistically significant ($P > 0.05$)

Table 1
Relative frequency of clinical signs accompanying retention of fetal membranes in ewes (in flocks in Southern Greece)

| Clinical sign | Ewes (*n*) showing the symptom | |
|---|---|---|
| | With intrauterine contamination | Without intrauterine contamination |
| Straining | 16 | 4 |
| Vulval edema and reddening | 11 | 4 |
| Anorexia | 11 | 1 |
| Recumbency | 11 | 1 |
| Increased temperature | 10 | 1 |
| Vaginal hyperemia | 2 | 4 |
| Cervical reddening and slightly rough shape | 5 | 0 |
| Increased uterine exudates | 5 | 0 |
| Watery vaginal/uterine discharge | 4 | 0 |
| Small clumps of white exudates | 3 | 0 |
| Cervical irregular and rough shape | 3 | 0 |
| Mucopurulent discharge | 3 | 0 |
| Purulent discharge | 3 | 0 |
| Depression | 2 | 0 |
| Cervical reddened and lacerated appearance | 1 | 0 |

Table 2
Bacterium or combination of bacteria from the uterine content of ewes with or without retention of fetal membranes (in flocks in Southern Greece)

| Bacterium or combination of bacteria | Ewes (*n*) from which isolated at | |
|---|---|---|
| | 1st sampling | 2nd sampling |
| From ewes with retention of fetal membranes | | |
| *A. pyogenes* | 8 | 11 |
| *E. coli* | 7 | 9 |
| *Aerobacter* sp. | 2 | 1 |
| *Staphylococcus simulans* | 2 | 4 |
| *Proteus mirabilis* | 1 | 0 |
| *Staphylococcus saprophyticus* | 1 | 1 |
| *Streptococcus uberis* | 1 | 2 |
| *Staphylococcus epidermidis* | 0 | 4 |
| *Streptococcus faecalis* | 0 | 3 |
| *Staph. aureus* | 0 | 2 |
| *A. pyogenes* + *E. coli* | 0 | 3 |
| *A. pyogenes* + *Staph. epidermidis* | 0 | 1 |
| *Aerobacter* sp. + *E. coli* | 0 | 1 |
| Total | 22 (24%) | 42 (46%) |
| From ewes without retention of fetal membranes | | |
| *E. coli* | 3 | 1 |
| *A. pyogenes* | 1 | 0 |
| *Staphylococcus epidermidis* | 1 | 0 |
| *Streptococcus faecalis* | 1 | 0 |
| *Streptococcus uberis* | 1 | 1 |
| Total | 7 (8%) | 2 (2%) |

change in the prevalence of intrauterine contamination from the first to the second sampling, among ewes without retention.

Among the 43 ewes with intrauterine contamination, retention was accompanied with other clinical signs in 22 (51%); among the 49 without, it was accompanied with other clinical signs in only 8 (16%). This difference was statistically significant ($P = 0.0007$). The frequency of clinical signs accompanying retention in ewes with or without intrauterine contamination is shown in Table 1. We found no association between the frequency of each clinical sign accompanying retention and the organism isolated from the uterine sample of that ewe ($P > 0.05$).

The principal bacteria isolated from the ewes with retention of fetal membranes were *A. pyogenes* (36% of the isolates on the first sampling, 32% on the second) and *E. coli* (32 and 26%, respectively). We found no statistically significant ($P > 0.05$) difference in the prevalence of intrauterine contamination with *A. pyogenes* or *E. coli* between the two samplings. Details of the identity of the isolated bacteria are in Table 2.

### 3.2. Antimicrobial agents susceptibility

*Staph. aureus* NCTC6571 was susceptible to all antibiotics tested. Of the 73 isolates tested, 15 (21%) were resistant to at least one and seven (10%) to two antimicrobial agents.

Table 3
Results of antimicrobial agents susceptibility tests of bacteria from the uterine content of ewes with or without retention of fetal membranes (in flocks in Southern Greece)

| | AM | CC | ENO | E | GM | N | P | TE |
|---|---|---|---|---|---|---|---|---|
| *A. pyogenes* ($n = 24$) | | | | | | | | |
| S | 22 | 24 | – | 22 | – | – | 22 | 24 |
| MS | – | – | – | – | – | – | – | – |
| I | 0 | 0 | – | 0 | – | – | – | 0 |
| R | 2 | 0 | – | 2 | – | – | 2 | 0 |
| *E. coli* ($n = 24$) | | | | | | | | |
| S | 22 | – | 22 | – | 24 | 22 | – | 22 |
| MS | 0 | – | – | – | – | – | – | – |
| I | – | – | 0 | – | 0 | 0 | – | 0 |
| R | 2 | – | 2 | – | 0 | 2 | – | 2 |
| *Staphylococcus* sp. ($n = 16$) | | | | | | | | |
| S | 14 | 16 | 16 | 15 | 16 | – | 14 | 15 |
| MS | – | – | – | – | – | – | – | – |
| I | – | 0 | 0 | 0 | 0 | – | – | 0 |
| R | 2 | 0 | 0 | 1 | 0 | – | 2 | 1 |
| *Streptococcus* sp. ($n = 9$) | | | | | | | | |
| S | 9 | 9 | – | 9 | – | – | 9 | 9 |
| MS | 0 | – | – | – | – | – | 0 | – |
| I | – | 0 | – | 0 | – | – | – | 0 |
| R | 0 | 0 | – | 0 | – | – | 0 | 0 |

Notes. AM, ampicillin (10 μg); CC, clindamycin (2 μg); ENO, enrofloxacin (5 μg); E, erythromycin (15 μg); GM, gentamicin (10 μg); N, neomycin (30 μg); P, penicillin-G (10 i.u.); TE, tetracycline (30 μg); S, susceptible; MS, moderately susceptible; I, intermediate; R, resistant; –, not tested.

The antimicrobial agents susceptibility pattern was not different in case of isolates obtained in the first or the second sampling from the same ewe. Details are present in Table 3.

### *3.3. Epidemiological associations*

In the first sampling, there were no associations between the odds of an ewe having an intrauterine contamination and the median clinical score or the median duration of retention in that flock.

In the second sampling, the odds of an ewe having an intrauterine contamination were multiplicatively increased by 1.06 (95% confidence interval: 1.02–1.13) when the median duration of retention in the flock increased by 6 h. There was no association between the odds of an ewe having an intrauterine contamination and the median clinical score of its flock.

The mean deviances of all models were close to one; therefore, no adjustment for over-dispersion was necessary.

## 4. Discussion

Postpartum metritis is of significant importance in cows. Affected animals develop a variety of genital and possibly generalized signs (''septic–toxic'' metritis). Their reproductive function is adversely affected and they often develop pyometra as a sequela to the infection [14].

Retention of fetal membranes is a major factor predisposing cows to postparturient uterine infection. Similar investigations have not been published in relation to ewes; this paper presents a part of the results of a larger study on the disorder in sheep. Other facets of this work have already been published [7,15,16].

We found that retention of fetal membranes clearly predisposed ewes to an increased prevalence of postpartum intrauterine contamination. Furthermore, the prevalence of intrauterine contamination in ewes with retention increased significantly as the puerperium advanced and it was positively associated with the duration of the disorder. This is not surprising, as retention has been found to delay the involution process of the genital system [17]; consequently the cervix remains open for a longer period and chances of intrauterine contamination are perennial. The retained membranes themselves provide a link from the uterine content to the outside environment, therefore, enhancing the chances of bacterial contamination.

In previous studies, dystocia and ketosis have been found to predispose cows to postpartum metritis [5,18]. We have already identified obstetrical assistance at lambing and energy insufficiency secondary to multiple pregnancy [15] as risk factors for retention in ewes and consequently, for postpartum intrauterine contamination.

Gunnick [19] suggested that cows with retention had an impaired leukocytic function. Inadequate supply of energy in relation to demand predisposes to an impaired leukocytic activity [20,21]. Since we have already established an association between retention and multiple-bearing pregnancies, which usually result in energy-deficient animals [22], we may postulate that ewes with retention also have an impaired leukocytic function. The

compromised immunological status of the uterus of a ewe with dystocia or multiple-bearing pregnancy, and the simultaneous retention may easily enhance susceptibility to intrauterine contamination.

Numerous microbial organisms have been isolated from the postparturient bovine uterus in cases of retention; the main ones were *A. pyogenes*, *E. coli*, *Staph. aureus* and anaerobes (review by Montes and Pugh [5]). The aerobic bacterial species isolated in our work were, in general, similar to those isolated from cows with retention of fetal membranes.

Although Steffan et al. [23] did not isolate anaerobes from cases of bovine postparturient metritis, in the majority of published articles, these are considered of increased importance in the pathogenesis of postpartum metritis [5,14,17,24,25]. Our results did not reveal anaerobic bacteria in the uterine samples examined. However, there is always the possibility that some anaerobic bacteria (e.g., *Bacteroides melaninogenicus*, *Fusobacterium necrophorum*) would not grow onto the media used.

We encountered limited problems in relation to antimicrobial agents sensitivity. Since the incidence risk of retention of fetal membranes is small and the clinical disorders associated with the condition subside quickly without life-threatening consequences, antimicrobial treatments are rarely carried out. Therefore, bacteria associated with this disorder would not be exposed frequently to the antimicrobial agents and would, consequently remain susceptible to these.

Fthenakis [26] and Saratsis et al. [27] examined the antimicrobial agents susceptibility rates of bacteria (*Staphylococcus* sp., *Streptococcus* sp., *E. coli*, *A. pyogenes*) from cases of ovine mastitis in the same farms. Their results showed susceptibility rates in older generation antimicrobial agents smaller than 20–50%; multi-resistant strains have also been isolated.

This sharp contrast between isolates from intrauterine contamination or mastitis may be explained by the facts that mastitis is more prevalent (incidence risk up to 30%; [28,29]) than postparturient intrauterine contamination, and that farmers and veterinarians frequently administer various antibiotics for treatment of the former disorder, while leaving the latter to self-cure.

It is noteworthy that high antimicrobial agents susceptibility rates of bacteria from cases of postparturient bovine metritis have been found by Cohen et al. [30], Steffan et al. [23] and Ziv [31].

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