



Prevalence of Bacterial Species Isolated from Subclinical Mastitis in Ewes in Siirt Province

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The aim of this study is to determine the prevalence of subclinical mastitis and the causative microorganisms in ewes in Siirt province, where small ruminant farming is widespread. The study used 349 ewes between days 50 and 80 of lactation. Milk samples were taken from the ewes and subjected to the California Mastitis Test (CMT). Scoring was performed according to the gelling stage. Milk samples were collected from ewes into sterile plastic tubes for somatic cell count (SCC) and bacterial isolation. Somatic cell count measurements were performed using the cell counter device. Bacterial analyses were carried out in the microbiology laboratory. In total, 349 sheep were evaluated, of which 198 (56.73%) tested positive for CMT and 151 (43.27%) tested negative. Furthermore, while bacterial growth was not detected in 145 (41.55%) of the 349 ewes, it was detected in 204 (58.45%). Coagulase-negative *Staphylococcus* (n:119, 58.33%) was the most frequently detected, followed by *Mycoplasma agalactiae* (n:35, 17.16%). Following these bacteria, *Escherichia coli* (n:15, 7.35%), *Klebsiella pneumoniae* (n:10, 4.90%), *Staphylococcus aureus* (n:10, 4.90%), *Enterococcus* spp. (n:5, 2.45%), Non-enterobacteriaceae (n:5, 2.45%), *Corynebacterium* spp. (n:2, 0.98%), *Micrococcus* spp. (n:2, 0.98%), and *Streptococcus* spp. (n:1, 0.49%) were isolated. In conclusion, a high prevalence of subclinical mastitis was found in Siirt province. It was concluded that informing farmers about the implementation of mastitis prevention and control programs would be beneficial.

Key Words: Mastitis, prevalence, ewe

Siirt Bölgesindeki Koyunlarda Subklinik Mastitisten İzole Edilen Bakteri Türlerinin Prevalansı

Bu çalışmanın amacı, küçükbaş hayvan yetiştiriciliğinin yaygın olduğu Siirt bölgesindeki koyunlarda subklinik mastitisin yaygınlığını ve etken mikroorganizmalarını belirlemektir. Çalışmada, laktasyonun 50 ile 80. günleri arasında olan 349 koyun kullanılmıştır. Koyunlardan süt örnekleri alınmış ve California Mastitis Testi'ne (CMT) tabi tutulmuştur. Puanlama, jelleşme durumuna göre yapıldı. Koyunlardan alınan süt örnekleri, somatik hücre sayımı (SHS) ve bakteri izolasyonu için steril plastik tüplere alındı. Cihaz kullanılarak somatik hücre sayımı ölçümleri yapıldı. Bakteri analizleri mikrobiyoloji laboratuvarında gerçekleştirildi. Toplamda 349 koyun değerlendirildi; bunların 198'inde (%56.73) CMT pozitif, 151'inde (%43.27) ise negatif sonuç elde edildi. Ayrıca, 349 koyunun 145'inde (%41.55) bakteri üremesi tespit edilmezken, 204'ünde (%58.45) tespit edildi. En sık tespit edilen mikroorganizma Koagülaz Negatif Stafilokoklar (n:119, %58.33) olurken, ikinci sırada *Mycoplasma agalactiae* (n:35, %17.16) yer aldı. Bu bakterileri takiben *Escherichia coli* (n:15, %7.35), *Klebsiella pneumoniae* (n:10, %4.90), *Staphylococcus aureus* (n:10, %4.90), *Enterococcus* spp. (n:5, %2.45), Enterobacteriaceae dışı bakteriler (n:5, %2.45), *Corynebacterium* spp. (n:2, %0.98), *Micrococcus* spp. (n:2, %0.98) ve *Streptococcus* spp. (n:1, %0.49) izole edildi. Sonuç olarak, Siirt bölgesinde subklinik mastitisin yüksek oranda görüldüğü tespit edilmiştir. Bu bağlamda çiftçilerin mastitis önleme ve kontrol programlarının uygulanması konusunda bilgilendirilmesinin faydalı olacağı sonucuna varılmıştır.

Anahtar Kelimeler: Mastitis, prevalans, koyun

Introduction

Mastitis is a disease characterized by inflammatory reactions in the mammary gland and changes in the physical and/or chemical structure of milk. Bacteria are the main cause of mastitis, but viruses, yeasts, and fungi also cause it (1, 2). There are two types of mastitis: Clinical mastitis, which causes varying degrees of clinical symptoms in the udder lobes and milk, and subclinical mastitis, which does not cause any clinical symptoms or physical changes in the milk (3, 4). In both forms of mastitis, there is a significant decrease in milk yield. Mastitis can cause a variety of economic losses, including a decrease in milk production, a decrease in milk quality, the cost of veterinary services, the early removal of mastitis-affected animals from the herd, additional costs from control and protection measures, and even economic losses due to a decrease in lamb nutrition/growth (5-7).

Gram-positive bacteria are considered the primary causative agents of mastitis in ewes (3). Most cases of clinical mastitis occur during the early stages of lactation, and researchers commonly report *Staphylococcus aureus* (*S. aureus*) and *Mannheimia hemolytica* as the major pathogens associated with the disease (8, 9). Clinical mastitis

accounts for less than 5% of all mastitis cases in sheep (10). The condition generally appears sporadically within flocks and only rarely manifests as herd-level outbreaks.

The diagnosis of clinical mastitis is relatively straightforward, as evident clinical signs can be observed both in the mammary gland and in the milk. Affected animals typically exhibit discoloration and swelling of the udder, accompanied by fever and pain. In many cases, sheep become lethargic, refuse to eat, and do not allow their lambs to suckle, resulting in reduced growth rates in the offspring. Additionally, significant alterations in the appearance and composition of milk are noted in sheep with clinical mastitis. These changes may include abnormal discoloration, the presence of blood, and serum-like secretions (11-14). In contrast to clinical mastitis, subclinical mastitis is highly prevalent in sheep. Coagulase-negative *Staphylococci* (CoNS) constitute the most frequently isolated bacterial agents, and herd-level prevalence has been reported to range from 25% to 90% (3, 9, 15). *Staphylococcus epidermidis* (*S. epidermidis*) is the second most isolated species (16). Following this, *Staphylococcus simulans* (*S. simulans*), *Staphylococcus chromogenes*, and *Staphylococcus xylosum* are identified. Less common species include *Staphylococcus caprae*, *Staphylococcus auricularis*, *Staphylococcus haemolyticus*, *Staphylococcus cohnii*, *Staphylococcus capitis*, *Staphylococcus equorum*, *Staphylococcus sciuri*, *Staphylococcus hominis*, *Staphylococcus muscae*, *Staphylococcus lentus*, *Staphylococcus saprophyticus*, and *Staphylococcus warneri* (9). *Staphylococcus aureus* is the most frequently isolated bacterium after CoNS in milk samples with subclinical mastitis (16). Antibiotic-resistant strains of *S. aureus*, especially methicillin-resistant *S. aureus*, are common worldwide and have also been reported in sheep (17).

This study aimed to determine the prevalence of bacterial species isolated from milk samples collected from sheep farms in Siirt province in Türkiye.

Materials and Methods

Research and Publication Ethics: Ethical approval for this study was obtained from the Siirt University Local Ethics Committee for Animal Experiments (07. 04. 2025 and 2025/03/08).

Animals: In this study, milk samples collected from ewes farms in the Siirt province were subjected to bacteriological analysis. A total of 349 ewes were included in the investigation. The animals were hand-milked during the lactation period, which ranged from 50 to 80 days.

California Mastitis Test: We performed the California Mastitis Test (CMT) on milk samples taken from ewes. We expressed and removed the first three streams of milks from each lobule before performing this test. We took a milk sample from each udder lobe into a separate compartment of the CMT cup for the CMT. Next, we added an equal amount of CMT solution with milk to each part of the container. We made circular

movements in a horizontal position for 10-15 seconds to allow the solution to interact with the milk. The same person then scored the CMT based on the gel consistency. According to score described by Schalm et al. (18), the results were divided into CMT negative (-) and positive (+) (within degrees of +, ++, and +++). If at least one mammary quarter received one of the values +, ++, and +++, the sheep was considered positive for CMT. If both udder lobes were negative, CMT was considered negative (19).

Somatic Cell Count: The somatic cell count (SCC) as measured using a DeLaval Cell Counter (Cellcounter DCC; DeLaval, Sweden) within one hour after the milk sample was collected from sheep. As described by Jaeger et al. (20), 60 µL of milk was aspirated into a cassette containing a specific DNA binding agent that identifies somatic cell nuclei. After the milk was aspirated, the cassette was inserted into the device, and the SCC in the aspirated milk was determined with a laser reader integrated into the device.

Milk sample collection, bacterial isolation, and identification: The udders of the ewes were cleaned thoroughly. After disinfection with a cotton swab soaked in 70% alcohol, milk samples (approximately 2 mL) were aseptically collected into sterile plastic tubes for microbiological analysis (21). The samples were transported to the Microbiology Laboratory of the Veterinary Faculty at Siirt University, and kept at + 4 °C during transport and delivered within a maximum of two hours. Bacteriological analyses were carried out in accordance with established standards (22). MacConkey plates, Sabouraud dextrose agar, defibrinated sheep blood, and 100 µL of each milk sample were added to blood agar plates (Bacto-Agar, Difco Laboratory). After 24 to 72 hours of incubation at 37 °C, the growth of each plate was evaluated. Initially, bacterial species were categorized using Gram staining and colony shape.

Catalase, tube coagulase, and fermentation tests for the generation of acid from glucose, mannitol, and maltose were used to identify the staphylococcal isolates as *S. aureus* and CoNS. Catalase and oxidase tests, growth on MacConkey agar, the IMVIC test, and the presence of a metallic sheen on EMB agar were used to identify *E. coli*. *Pseudomonas* spp. were recognized by oxidase reaction and growth on MacConkey agar; *Corynebacterium* spp. were identified by hemolysis, nitrate reduction, and urease activity; and *Bacillus* spp. were identified by significant hemolysis and endospore development. Cellular organization and oxidation/fermentation assays were used to identify *Micrococcus* spp.

Milk samples were cultured on agar plates containing selective medium and incubated at 37°C in a 5% CO₂ environment for 3 to 4 days. Following incubation, colonies exhibiting the *Mycoplasma agalactiae* (*M. agalactiae*) were identified utilizing an optical microscope at 100 × magnification (9, 23, 24).

As illustrated in Figure 1, identification of *S. aureus* isolates was performed by PCR based on amplification

of the *S. aureus* Coa gene reported by Schmitz et al. (25) and the nuc gene reported by Brakstad et al. (26). Identification of *M. agalactiae* isolates was performed by PCR as reported by Tola et al. (27) (Table 1).

Statistical Analysis: The normality of the distributions was tested in the SCC levels using visual methods (probability plots and histograms) and the Shapiro-Wilk test. According to the tests, the data did not show a normal distribution. Therefore, the Kruskal-Wallis test, which is a nonparametric test, was used for intergroup comparisons for all parameters. The post-hoc pairwise analysis also included the Mann-Whitney-U test with Bonferroni adjustment ($p < 0.05$).

Results

Out of 349 ewes used in the study, 198 ewes (56.73%) showed a positive CMT result in at least one mammary lobe, and 151 ewes were identified as CMT (-) (43.27%). In addition, although 145 (41.55%) of the total 349 ewes milk samples did not have bacterial growth, it was found that 204 (58.45%) milk samples had bacterial growth (Table 2).

In these 204 milk samples, the highest percentage of CoNS (n:119, 58.33%) was isolated. After CoNS, the second most frequently isolated bacterium was *M. agalactiae*, accounting for 35 cases (17.16%). Following these bacteria, *Escherichia coli* (*E. coli*) (n: 15, 7.35%), *Klebsiella pneumoniae* (*K. pneumoniae*) (n: 10, 4.90%), *S. aureus* (n: 10, 4.90%), *Enterococcus* spp. (n: 5, 2.45%), *Non-enterobacteriaceae* (n: 5, 2.45%), *Corynebacterium* spp. (n: 2, 0.98%), *Micrococcus* spp. (n: 2, 0.98%), and *Streptococcus* spp. (n: 1, 0.49%) were isolated (Table 3).

Somatic cell count was measured on 349 milk samples. When we look at the mean SCC of milk samples, it was calculated as 140.00 ± 70.32 ($\times 10^3$ cells/mL) in non-growth bacteria (n: 145), CoNS (n:119, $815.00 \pm 41.66 \times 10^3$ cells/mL), *M. agalactiae* (n: 35, $880.00 \pm 89.14 \times 10^3$ cells/mL), *E. coli* (n: 15, $1430.00 \pm 186.08 \times 10^3$ cells/mL), *K. pneumoniae* (n: 10, $820.00 \pm 75.67 \times 10^3$ cells/mL), *S. aureus* (n: 10, $1120.00 \pm 98.75 \times 10^3$ cells/mL), and other bacteria (*Enterococcus* spp, *Non-enterobacteriaceae*, *Corynebacterium* spp, *Micrococcus* spp, *Streptococcus* spp) (n: 15, $789,00 \pm 53.93 \times 10^3$ cells/mL) (Table 4).

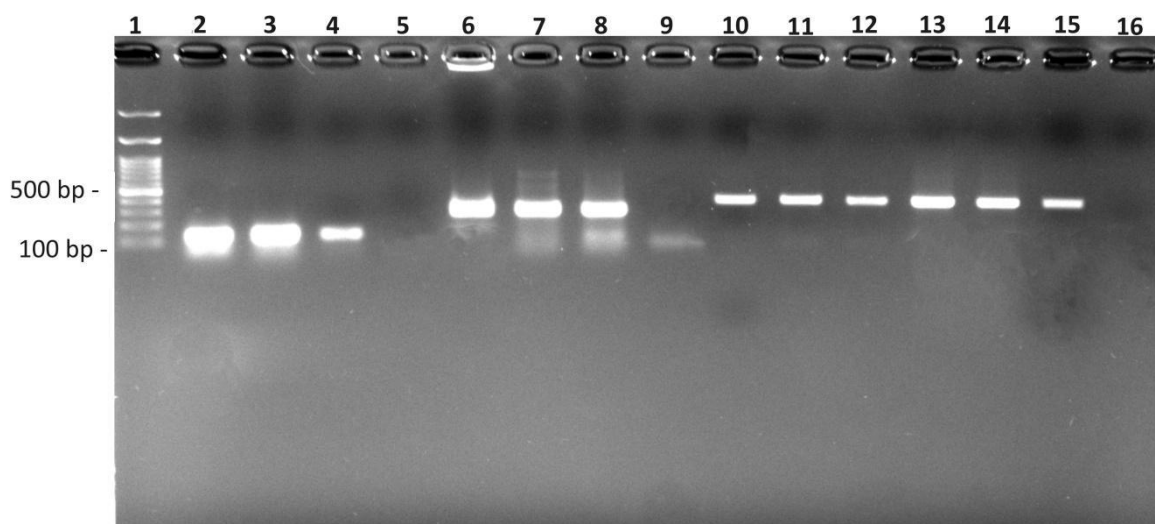


Figure 1. Agarose gel image of amplicons obtained from samples in which *S. aureus* and *M. agalactiae* were identified by PCR (1:100 bp DNA Ladder, 2-4: *S. aureus* coa gene (131 bp), 5: Negative Control; 6-8: *S. aureus* nuc gene (270 bp), 9: Negative Control; 10-15: *M. agalactiae* (375 bp), 16: Negative Control).

Table 1. Primer sequences used in the PCR identification of *S. aureus* and *M. agalactiae* isolates in mastitis milk samples examined in the study

Gene	Oligonucleotide (5'-3')	Amplicon Size (bp)	Reference
Coa	F: GCTTCTCAATATGGTCCGAG	131 bp	Schmitz et al. (25)
	R: CTTGTTGAATCTTGGTCTCTCGC		
Nuc	F: GCGATTGATGGTGATACGGTT	270 bp	Brakstad et al. (26)
	R: AGCCAAGCCTTGACGAACAAAGC		
<i>M. agalactiae</i>	F: AAAGGTGCTTGAGAAATGGC	375 bp	Tola et al. (27)
	R: GTTGCAAGAAAGTCCAATCA		

Table 2. Bacterial growth rates according to CMT score

CMT	Bacterial growth					
	Ewes		Growth bacteria		Non-growth bacteria	
	n	%	n	%	n	%
CMT negative	151	43.27	11	5.39	140	96.55
CMT positive	198	56.73	193	94.61	5	3.45
Total	349	100.00	204	100.00	145	100.00

Table 3. Bacterial isolates from milk samples (n: 204) in ewes with subclinical mastitis

Bacteria	n (%)
Coagulase-negative <i>Staphylococci</i>	119 (58.33%)
<i>Mycoplasma agalactiae</i>	35 (17.16%)
<i>Escherichia coli</i>	15 (7.35%)
<i>Klebsiella pneumoniae</i>	10 (4.90%)
<i>Staphylococcus aureus</i>	10 (4.90%)
<i>Enterococcus</i> spp.	5 (2.45%)
Non-enterobacteriaceae,	5 (2.45%)
<i>Corynebacterium</i> spp.	2 (0.98%)
<i>Micrococcus</i> spp.	2 (0.98%)
<i>Streptococcus</i> spp	1 (0.49%)
Toplam	204 (58.45%)

Table 4. Somatic cell count in milk samples

Bacterial growth	n	SCC (x103 cell/mL)	
		Median ± Std. Error of Mean	Minimum-Maximum
Coagulase-negative <i>Staphylococci</i>	119	815.00±41.66 ^b	120.00-5083.00
<i>Mycoplasma agalactiae</i>	35	880.00±89.14 ^b	371.00-3382.00
<i>Escherichia coli</i>	15	1430.00±186.08 ^c	671.00-5246.00
<i>Klebsiella pneumoniae</i>	10	820.00±75.67 ^b	245.00-1743.00
<i>Staphylococcus aureus</i>	10	1120.00±98.75 ^c	633.00-1348.00
Other bacteria (<i>Enterococcus</i> spp, Non-enterobacteriaceae, <i>Corynebacterium</i> spp, <i>Micrococcus</i> spp, <i>Streptococcus</i> spp,)	15	789.00±53.93 ^b	220.00-4176.00
Non-growth bacteria	145	140.00±70.32 ^a	86.00- 510.00
<i>p</i>		0.002	

^{a, b, c}: The difference between groups with different superscripts in the same column is statistically significant, ($p < 0.05$).

Discussion

Mastitis in sheep is mostly caused by Gram-positive bacteria (3). Most cases of clinical mastitis occur in the early stages of lactation and are reported to be caused mostly by *S. aureus* and *Mannheimia haemolytica* (8, 9). Less than 5% of sheep incidences of mastitis often result in clinical mastitis (10). However, clinical mastitis is seen sporadically in sheep. Although rare, it can occur in the form of herd outbreaks. As the

severity of the disease increases, clinical mastitis may transition from subacute to chronic. Clinical mastitis is straightforward to identify because it presents with physical symptoms such as udder discolouration as well as easily observable clinical indicators. Clinical mastitis typically results in swollen, painful udder lobes. Affected ewes refuse to feed, are lethargic and often do not allow their lambs to suckle, resulting in reduced growth rates in suckling lambs. Milk from sheep infected with clinical mastitis has an unusual appearance and composition; it

may be discolored, watery, or contain blood or serum (11-14).

Subclinical mastitis is one of the most serious diseases that cause economic losses in the mammary glands of goats and sheep worldwide. The disease is characterized by the development of intramammary infection without clinical symptoms (28). Subclinical mastitis can be diagnosed through bacteriological tests or SCC in milk (neutrophil leukocytes and some epithelial cells) (10, 28).

It is stated that the prevalence of subclinical mastitis, which is quite common in small ruminants, varies between 6.5% and 40.2% (29, 30, 31). In a study conducted in Western Algeria, the prevalence of SCM among 150 goats was reported to be 20.7% (32). In the Laghouat area, a higher prevalence of 46.6% was observed in 60 local goats (33). In our study, 58.45% of subclinical mastitis cases were observed in 349 sheep according to the CMT score. The types of bacteria detected in subclinical mastitis milk and their isolation rates may vary depending on many factors such as milking techniques of animals, hygiene and sanitation methods applied, and growing conditions (34, 35). Ergene et al. (36) detected 27.3% bacterial growth in their study using 227 milk samples in Cyprus. Ergun et al. (37) found a lower rate of bacteriological growth (6.4%) in milk samples. Bergonier et al. (3) and Contreras et al. (8) found that the prevalence of subclinical mastitis ranged between 5% and 30%. In this study, bacterial growth was detected in 59.10% of the milk samples obtained from sheep.

Many researchers have claimed that CoNS is the most often identified bacteria in milk samples (38-41). In sheep, CoNS are among the most commonly isolated bacterial species in subclinical mastitis, with a prevalence ranging from 25% to 90% at the herd level (3, 9, 15, 41). Recent studies indicate the importance of CoNS as etiological agents of subclinical mastitis in sheep (8, 42). Zafalon et al. (41) found the frequency of CoNS in 56.8% of bacteriology positive samples. In the study conducted by Vasileiou et al. (34), CoNS was detected in 59.7% of subclinical ewes mastitis cases. Contrary to these studies, Gökhan and Gülaydın (43) reported that they found 3% CoNS (*S. simulans* and *S. epidermidis*) in 103 milk samples. In this study, the most commonly isolated bacteria were CoNS with 58.33%.

The most frequently isolated bacterium after CoNS in subclinical mastitis is *S. aureus* (16). Ergün et al. (37) reported that they found *S. aureus* at a rate of 3.1% in subclinical mastitis samples. Kern et al. (44) reported that *S. aureus* was detected in 5.5% of the isolated

bacteria, while Zafalon et al. (41) identified it in 8.1% of bacteriology-positive milk samples. Tancin et al. (45) reported that they detected 3.33% *S. aureus* in a study conducted on 116 milk samples. Holko et al. (46) detected the incidence of *S. aureus* in 6.9% of milk samples. In this study, *S. aureus* was detected in 4.90% of 204 milk samples.

On the other hand, mycoplasmas are microorganisms that cause chronic infections that are difficult to eradicate, with complex unknown pathogenicity factors. Mycoplasma-induced mastitis continues to be a significant problem worldwide. *Mycoplasma bovis* is the most significant species in cattle, whereas its very close phylogenetic relative, *M. agalactiae*, causes very severe mastitis in small ruminants. *Mycoplasma agalactiae* is the main etiological agent of contagious agalactiae syndrome in sheep and goats and causes significant economic losses, especially as the infection lingers in milk for many years even after antibiotic treatment (47-49). In their study, Göçmen et al. (50) found *M. agalactiae* positive in 9.14% of 339 samples. Similar to these results, in our study, *M. agalactiae* was detected as the second most isolated bacterial agent with a rate of 17.16%.

In their study conducted in Sharkia province of Egypt, Abdallah et al. (28) reported that the most common bacterial species isolated from sheep milk samples was *E. coli* (44.4%). In a study conducted by Ergün et al. (37) on sheep with mastitis, 2% of 1458 milk samples were reported to contain *E. coli*. In a study conducted on goats by Doğruer et al. (51), the *E. coli* rate was determined as 4.5%. In our study, *E. coli* (7.35%) was detected, similar to this study. The reason for this wide range of *E. coli* is that it is an environmental microorganism and is affected by factors such as poor ventilation, inadequate manure removal and general lack of cleanliness and sanitation on the farm.

As a result, it is seen that subclinical mastitis is an important health problem in Siirt province. In this study conducted on Hamdani ewes, 58.45% subclinical mastitis was reported. In the study on the isolation of the agent causing subclinical mastitis, it was determined that CoNS had the highest isolation rate. Following this, *M. agalactiae*, detected by molecular methods, was found to be in second place with 17.16%. In order to prevent and combat mastitis, it is thought that it would be beneficial to inform breeders in Siirt province about this issue and to provide the necessary incentives to ensure they pay attention to milking hygiene and have a healthier lactation period.

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