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ORIGINAL ARTICLE

Caprine Subclinical Mastitis: Prevalence and Etiological Study in Central Iran

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Abstract

Subclinical mastitis is a significant problem in dairy goat husbandry and has a negative impact on milk production and animal health. This study investigated the microbial pathogens associated with subclinical mastitis in goats from Isfahan province, focusing on the identification of bacterial species, evaluation of their biofilm-forming ability, and assessment of their antibiotic resistance patterns. A total of 200 goats were randomly selected from three livestock-rich districts in Isfahan. Samples were collected from apparently healthy goats, and the Somatic Cell Count (SCC) was measured to assess udder health. Polymerase Chain Reaction (PCR) was used to detect pathogenic bacteria, including *Staphylococcus* spp., *Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus* spp. The results showed that 97% of the samples had an SCC value of less than 600,000 cells/mL. Of the 54 samples tested by PCR, 42.59% contained *Staphylococcus* genes, 25.92% contained *S. aureus*, and 14.81% contained both *Streptococcus* spp. and *E. coli*. In addition, 62.96% of the isolates formed moderate biofilms, while 22.22% exhibited strong biofilm formation. Alarmingly, all isolates showed resistance to amoxicillin, ciprofloxacin, and ceftriaxone. These results underline the need for improved hygiene practices on dairy farms and continuous monitoring of milk quality to effectively address the challenges posed by pathogenic bacteria and antibiotic resistance. In addition, the development of alternative therapeutic methods to treat resistant infections is also recommended.

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Introduction

In recent decades, goat breeding has experienced significant growth in many countries worldwide, including Asian countries, and Iran is no exception (1, 2). Among the most important reasons for the expansion of goat breeding are the increased demand for goat products, including milk and meat, to meet food needs, as well as the provision of financial support for local communities (3).

A critical problem for goat breeders is subclinical mastitis, a type of mammary gland inflammation that does not present apparent external symptoms such as redness or swelling and therefore significantly impacts herd health and goat trade (4). Changes in milk composition and elevated Somatic Cell Counts (SCC) are typically used to identify this illness. Significant reductions in milk yield and quality can result from the underlying condition, even if the goats appear clinically healthy (5,6).

Studies by Novotna et al. (2018) and Bogdanovičová et al. (2016) have demonstrated that goat milk typically contains high SCC levels, averaging between 1100 and $1300 \times 10^3/\text{ml}$ (7, 8). However, levels frequently rise beyond this baseline due to subclinical infections, leading to substantial economic losses. Therefore, investigating the underlying causes of these SCC levels and their potential effects on milk quality is essential.

The presence of high SCCs, even in apparently healthy (mastitis-free) goats, is a critical indicator of the suitability of milk for technological processing in dairies.

The main bacteria associated with subclinical mastitis in ruminants include *Streptococcus*, *Staphylococcus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Bacillus* species (4, 9, 10). The presence of pathogenic bacteria, in particular, can increase SCC levels, ultimately having a negative effect on milk quality (11). These bacteria can be transferred from the environment or infectious sources and may lead to a significant decrease in milk production, animal weakness, and even reproductive problems (12).

Furthermore, bacterial biofilm formation plays a major role in the development of chronic infections, such as subclinical mastitis in goats (13, 14). On a variety of surfaces, biofilms develop as microbial communities that make bacteria more resistant to antibiotics and host immune responses. Certain bacteria can easily form biofilms in the mammary glands, leading to chronic infections that adversely affect milk quality (15, 16).

The identification of the causative pathogens, mainly bacterial pathogens, has played a central role in the development of targeted antimicrobial treatments and

preventive management strategies (17). Although bacterial culture of animal milk samples is the gold standard for the diagnosis of intramammary infections in small ruminants, DNA-based molecular typing methods are more efficient and demonstrate high sensitivity and specificity for the identification of microorganisms (18). Therefore, molecular techniques, specifically polymerase chain reaction (PCR), were used to identify these pathogens in the present study.

Considering the above information, the aim of the present study was to identify the microbial pathogens responsible for subclinical mastitis in goats in Isfahan province, investigate their biofilm-producing ability, and evaluate their antibiotic resistance patterns.

Material and Method

Sample Collection

For this study, three livestock-rich counties, Najafabad, Khomeini Shahr, and Tirran and Krun, were selected, characterized by a temperate and relatively dry climate. A total of 200 native goats (age: 2-5 years; parity: 1-4; 30-150 days in milk) were randomly sampled from five traditional farms in each county.

Sampling was conducted exclusively on healthy goats that exhibited no clinical signs of mastitis. The selected goats were milked by hand twice daily on average. Prior to sampling, one udder was randomly selected and cleaned using a sterile cloth, followed by washing with soap and water. The teat ends were subsequently disinfected with 70% ethanol. After allowing the teats to dry completely, several initial streams of milk were discarded to minimize contamination, and approximately 70 mL of milk was collected from each goat into sterile containers maintained on ice for transport to the laboratory. Ten milliliters of each sample were preserved at -20°C for subsequent PCR analysis.

SCC Analysis

To determine SCC, the FOSSOMATIC 500 Basic device (FOSS Analytics, Hillerød, Denmark) was utilized in the laboratory, employing a high-speed infrared spectrophotometric method. The SCC results were categorized according to the criteria established by Podhorecká et al. (2021) (19). In goat milk, the normal range of SCC values typically falls between 1000 and 1300×10^3 cells/mL. An SCC exceeding 600×10^3 cells/mL was considered an alert threshold, indicating potential microbial inflammation within the mammary glands.

Isolation and Culture of Bacteria from Samples with SCC Above and Below the Threshold

To confirm the presence of bacteria in the milk samples, both molecular and culture-based methods were utilized. All samples exhibiting SCC values above the established threshold, along with 51 samples below this threshold, were subjected to bacterial culture. For bacterial isolation, samples were cultured on blood agar and MacConkey agar (HiMedia, India) and incubated at 37°C for 24-48 hours under aerobic conditions. Samples showing mixed bacterial growth were re-streaked onto appropriate media to obtain pure colonies for subsequent identification and testing. Samples with no observable growth after the incubation period were considered culture-negative. The identification of bacterial species was performed using standard diagnostic criteria outlined in the Veterinary Diagnostic Bacteriology textbook (20).

PCR Testing for Pathogen Detection

For PCR testing, samples with positive SCC results were selected, along with additional samples having cell counts below 600×10^3 cells/mL to assess the presence of four

bacterial species: *E. coli*, *S. aureus*, *Staphylococcus* species, and *Streptococcus* species. Bacterial DNA was extracted directly from the goat milk samples using standard extraction protocols. Briefly, milk samples were centrifuged to precipitate bacterial cells. Following lysis of the cells, bacterial DNA was released and purified using a silica-based method with Tris-EDTA buffer at pH 8. The quantity and quality of the extracted DNA were assessed using a UV spectrophotometer (Q3000, Quawell, USA).

For pathogen identification, four primer pairs specific to the aforementioned bacteria were utilized in the PCR assays (Table 1). Each PCR reaction was performed in a total volume of 25 μ L containing 2.5 μ L of 10 \times PCR buffer, 25 ng of template DNA, 0.2 mM dNTPs, 2 mM MgCl₂, 0.4 μ M of each primer, and 0.05 units of Taq polymerase (TaKa, Dalian, China).

The thermal cycling conditions comprised an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 40 seconds, with a final extension at 72°C for 10 minutes. The amplified products were analyzed using electrophoresis on a 2% agarose gel to confirm the presence of target pathogens (21).

Table 1: Oligonucleotide Sequences and PCR Product Sizes of Various Microorganisms

Microorganisms	Primers name	Oligonucleotide sequences (5' to 3')	PCR product size (bp)
<i>E. coli</i>	<i>phoA for</i>	GGTAACGTTTCTACCGCAGAGTTG	468
	<i>phoA rev</i>	CAGGGTTGGTACTGTCATTACG	
<i>S. aureus</i>	<i>nuc for</i>	TTCGAAAGGGCAATACGCAAAGAGG	227
	<i>nuc rev</i>	TAACCGTATCACCATCAATCGCTTT	
<i>Staphylococcus spp.</i>	<i>gap for</i>	GCAGCAGCAGAAAACATCATCCCTA	318
	<i>gap rev</i>	TTGACGGTCGCCAACTGACATTACA	
<i>Streptococcus spp.</i>	<i>16SrRNA for</i>	ACCAACTGGATGAGCAGCGAACGGG	590
	<i>16SrRNA rev</i>	TCTCCTCTCCTGCACTCAAGTCTAC	

Assessment of Biofilm Formation

The ability of bacterial isolates to produce biofilms was evaluated using a microtiter plate assay combined with crystal violet staining, following protocols described in previous studies (22, 23). Uninoculated TSB was used as a negative control, and the cutoff optical density (OD_c) was defined as the mean optical density of the negative control plus its standard deviation. The biofilm-forming ability of

the tested isolates was categorized into four groups based on optical density: isolates with OD \leq OD_c were classified as non-adherent; those with OD between OD_c and 2 \times OD_c were identified as weak biofilm producers; isolates with OD between 2 \times OD_c and 4 \times OD_c were considered moderate biofilm producers; and those with OD > 4 \times OD_c were categorized as strong biofilm producers.

Antibiotic Susceptibility Testing

To assess the susceptibility of bacterial isolates to various antibiotics, a modified Kirby-Bauer disk diffusion method was employed in accordance with CLSI guidelines (24). Briefly, bacterial suspensions were adjusted to a 0.5 McFarland standard in Mueller–Hinton broth and subsequently spread onto Mueller–Hinton agar plates. Antibiotic disks were placed on the agar surface using sterile forceps. After a 24-hour incubation at 37°C, resistance or sensitivity was determined by measuring the zones of inhibition using a caliper. The antibiotics tested in this study included amoxicillin (25 µg per disk), ceftriaxone (30 µg per disk), ciprofloxacin (5 µg per disk), chloramphenicol (30 µg per disk), and oxytetracycline (30 µg per disk). All antibiotic disks were procured from Padtan Teb Company.

Results

A total of 54 bacterial isolates were obtained for biofilm testing. Of these, 24 isolates were selected for antibiotic susceptibility testing based on their clinical significance.

SCC

Out of a total of 200 goat milk samples referred to the laboratory, only six samples (3%) exhibited an SCC

exceeding 600,000 cells/mL. The remaining 197 samples (97%) demonstrated an SCC below 600,000 cells/mL.

Cultural and PCR Testing for Pathogen Detection

Based on the culture results, the identified bacteria included *E. coli*, *S. aureus*, and various species of *Staphylococcus* and *Streptococcus*. Among the 54 samples selected for this analysis, 23 samples (42.59%) were positive for genes associated with *Staphylococcus* species, 14 samples (25.92%) contained genes specific to *S. aureus*, 8 samples (14.81%) harbored genes related to *Streptococcus* species, and another 8 samples (14.81%) contained genes linked to *E. coli*. The band sizes and electrophoresis results for each of the respective genes are shown in Figure 1.

Capability of Biofilm Formation

Results obtained using the ELISA reader demonstrated that 53 out of 54 isolates (98.15%) were capable of biofilm formation to varying degrees. The biofilm production capacity of the isolates was categorized as follows: 7 samples (12.96%) produced weak biofilms, 34 samples (62.96%) formed moderate biofilms, and 12 samples (22.22%) exhibited strong biofilm production. Only one isolate (1.85%) showed no biofilm formation capability.

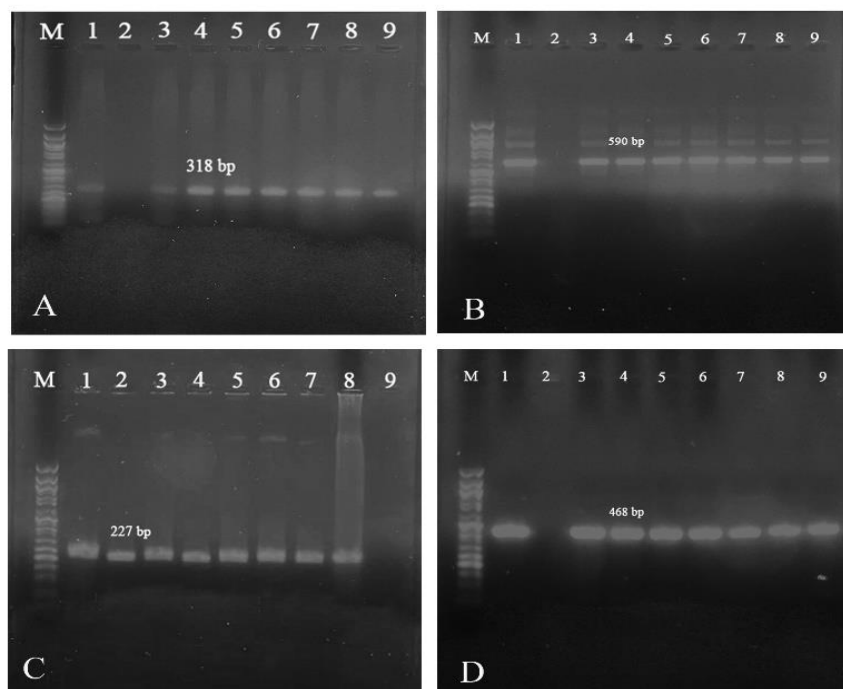


Figure 1: Electrophoresis results of PCR products amplifying the specific genes in milk samples. The *phoAT* gene of *E. coli* on a 2% agarose gel. (A) *Staphylococcus* spp. (B) *Streptococcus* spp. (C) *S. aureus* and (D) *E. coli*. Lane M: DNA ladder; Lane 1: positive control; Lane 2 (A, B, D) and Lane 9 (C): negative control; Lanes 3 to 8-9: selected isolates with positive reactions.

Antibiogram

Not all biofilm-tested isolates were subjected to antibiotic susceptibility analysis. The antibiotic testing was strategically performed on a subset of 24 isolates representing the most clinically relevant pathogens. The results of the antimicrobial susceptibility testing were summarized in Table 2. The findings indicated that all 24

isolates tested were resistant to amoxicillin, ciprofloxacin, and ceftriaxone. For oxytetracycline, 1 isolate (4.17%) was susceptible, 21 isolates (87.50%) showed intermediate susceptibility, and 2 isolates (8.33%) were resistant. Regarding chloramphenicol, 20 isolates (83.33%) were susceptible, 3 isolates (12.50%) exhibited intermediate susceptibility, and 1 isolate (4.17%) was resistant.

Table 2: Antimicrobial susceptibility results of bacterial isolates (N=24)

Antibiotic	Susceptible N (%)	Intermediate N (%)	Resistant N (%)
amoxicillin	0 (0)	0 (0)	24 (100)
ciprofloxacin	0 (0)	0 (0)	24 (100)
ceftriaxone	0 (0)	0 (0)	24 (100)
oxytetracycline	1 (4.17)	21 (87.50)	2 (8.33)
chloramphenicol	20 (83.33)	3 (12.50)	1 (4.17)

Discussion

Subclinical mastitis is a major problem in dairy goat husbandry, affecting both milk production and animal health (25). Therefore, the present study focused on Isfahan province and aimed to identify the bacterial species associated with mastitis, as well as their ability to form biofilms and their antibiotic resistance patterns.

The results of SCC analysis in goat milk samples indicate a favorable health status and high milk quality in the studied area. Only 3% of the samples had SCC values above 600,000 cells/ml, while 97% had SCC values below 600,000 cells/ml. These findings suggest that the animals were generally healthy and free of clinical signs of mastitis, likely due to proper dairy management and hygiene practices. However, the concurrent detection of biofilm-forming pathogenic bacteria in some samples suggests the possibility of early-stage infections in which the host inflammatory response has not yet been fully elicited, a factor that may not be immediately reflected in SCC values. This paradigm underscores the superior sensitivity of molecular methods in identifying latent threats to milk quality that SCC alone might miss. In a study conducted in the Savojbolagh region in Iran, Razapour et al. (2015)

investigated the effects of subclinical mastitis on SCC in Saanen goat milk and reported that, among 65 Saanen goats that had been lactating for more than one week, an increase in the severity of mastitis was associated with an increase in SCC levels (26). According to the study by Leitner et al. (2004), subclinical mastitis caused by coagulase-negative staphylococci led to a significant increase in SCC and reduction in milk production (27). Therefore, the association between subclinical mastitis and increased SCC supports SCC as a valuable indicator for the detection and monitoring of udder health, especially in cases where no overt symptoms are present.

The use of PCR techniques in this study enabled rapid and accurate identification of the mastitis-causing bacteria. The results show that *Staphylococcus*, *Streptococcus*, *E. coli*, and *S. aureus* are among the main causative agents of subclinical mastitis in dairy goats in Isfahan province. 42.59% of the goat milk samples contained genes related to *Staphylococcus* species, with 25.92% attributable to *S. aureus*. In addition, 14.81% of the samples each contained genes from *Streptococcus* and *E. coli*. These findings are consistent with a study by Zhao et al. (2015) in China, which reported that of 683 goats, 313 goats (45.82%) were diagnosed as positive or strongly positive for subclinical

mastitis using the California Mastitis Test. Of these positive goats, 209 milk samples were used to identify the pathogens by multiplex PCR. The results showed: coagulase-negative *Staphylococcus* (59.52%), *S. aureus* (15.24%), *E. coli* (11.43%), and *Streptococcus* species (10.95%), which is comparable to the results of our study (21).

Studies have shown that within the genus *Staphylococcus*, only *S. aureus* and coagulase-negative staphylococci can colonize the mammary glands of ruminants, potentially leading to subclinical mastitis (28, 29). Merz et al (2016) reported that 46% of tank milk samples from small ruminants tested positive for *S. aureus* (30). Similarly, Condas et al. (2017) found a prevalence of 43.1% for *S. aureus* in bulk tank milk samples (31). Cortimiglia et al. (2015) identified MRSA in 2 % of tank milk samples from dairy goat farms (32). Mastitis in goats is a common problem in dairy production worldwide, with *Staphylococcus aureus* being the most important pathogen (21). The worldwide prevalence of goat mastitis ranges from 5% to 70%. In certain European countries, such as Spain and Greece, the prevalence is estimated to be between 15 % and 30 % (33, 34). Praja et al. (2023) isolated *S. aureus* from 258 raw goat milk samples from several dairy farms (35). Recent reports indicate that *S. aureus* is a significant pathogen in the dairy industry and its products (36). The results of this study are consistent with similar studies in other countries and demonstrate that bacteria such as *Staphylococcus*, *Streptococcus*, *E. coli*, and *S. aureus* are common causative agents of subclinical mastitis in dairy goats. This underlines the need to implement control measures to reduce the contamination of milk by these bacterial pathogens.

The results of this study show that the majority of bacterial isolates obtained from goat milk samples have the ability to form biofilms. Approximately 62.96% of the isolates formed moderate biofilms, while 22.22% produced strong biofilms, indicating a high potential of these bacteria to cause chronic and treatment-resistant infections. Several studies have reported biofilm production rates for *S. aureus* ranging from 40% to 90%. A study conducted in Iran found that 76.6% of *S. aureus* strains had the ability to form biofilms (37). In the case of *E. coli*, studies have shown that 60 % of strains have the ability to form biofilms (38). Based on the biofilm formation rates observed in the isolates in the present study and their comparison with findings from other studies, it can be concluded that even the extent of biofilm formation by bacteria may play a role in the type of mastitis they cause, as well as their antibiotic resistance profile.

The prevalence of antibiotic-resistant bacteria in goat milk is a cause for concern. The results of the antibiogram tests in this study show a worrying level of antibiotic

resistance among bacterial isolates from goat milk samples. All 24 samples tested showed resistance to amoxicillin, ciprofloxacin, and ceftriaxone, which is likely due to the frequent and inappropriate use of these antibiotics on dairy farms. Several studies have also reported the presence of multidrug-resistant strains in raw, bulk, and pasteurized milk. Ranjbar et al. (2018) reported the contamination of milk from the northwestern provinces with toxin-producing, multidrug-resistant strains (39). Akindolire et al. (2015) identified MRSA in bulk tank milk from dairy goat farms in northern Italy (40). Rahi et al. (2020) emphasized that raw milk could serve as a source of multidrug-resistant MRSA (41). Regarding oxytetracycline, only one sample showed susceptibility, while 21 samples exhibited intermediate susceptibility. For chloramphenicol, 20 samples were sensitive, and 3 demonstrated intermediate sensitivity. These results suggest that these antibiotics could be potential therapeutic options but require further investigation. Given the state of antibiotic resistance, continuous monitoring of antibiotic use and the implementation of appropriate hygiene protocols on dairy farms are essential.

Conclusion

The results of this study indicate a good quality of goat milk in the province of Isfahan, as only 3% of the 200 samples analyzed had SCC values of more than 600,000, while the remaining samples were within acceptable limits. This finding clearly reflects compliance with hygiene practices during milking and storage. However, the detection of pathogenic bacteria such as *S. aureus*, *E. coli*, and other *Staphylococcus* and *Streptococcus* species in some samples, particularly in 42.59% of the samples, indicates the presence of potentially dangerous microbial pathogens that could affect milk quality and animal health. In addition, the biofilm-forming ability of the isolates and their resistance to antibiotics, in particular amoxicillin, ciprofloxacin, and ceftriaxone, raise serious concerns regarding infection control and therapeutic management.

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Authors' Contributions

Soroosh Hemati: Conceptualization, investigation; **Jalal Shayegh:** supervision, writing original draft, methodology, investigation, review & editing, formal analysis, visualization.

Data Availability

All data generated or analyzed during this study are included in this published article.

Ethical Approval

This study was reviewed and approved by the research ethics committee of the Faculty of Veterinary Medicine, Islamic Azad University of Tabriz, Iran.

Conflict of Interest

No potential conflicts of interest.

Consent for Publication

Not applicable

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