



The Frequency of Bacterial Pathogens of Mastitis and their Antibiotic Susceptibility in Saanen and Alpine Goats

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ABSTRACT

Background: The purpose of Saanen and Alpine raising farms is milk production, so control of mastitis is essential. Antibiotic resistance among mastitis pathogens has been increased by long-time usage of antibiotics. The aim of the present study was to isolate bacteria from mastitis cases in Saanen and Alpine goats and to determine their antibiotic resistance.

Methods: Milk sample of 26 Saanen and 29 Alpine was cultured on microbiological media. Polymerase chain reaction was performed for the detection of *Mycoplasma*. Antibiotic resistance of the bacteria was tested by 12 antibacterial disks. Significant difference in resistance levels between Saanen and Alpine was assessed.

Results: *Mycoplasma* spp. was detected in 12.73% of samples. The isolated bacteria were *Escherichia coli* (29.1%), *Trueperella pyogenes* (25.5%), *Staphylococcus aureus* (16.4%), *Streptococcus agalactiae* (9.1%), coagulase negative staphylococci (5.5%), and *Corynebacterium pseudotuberculosis* (1.8%). The significant difference was observed in antibiotic resistance to amoxicillin which was higher in Alpine.

Conclusion: Accordingly using antibiotics more accurate and using antibiogram by clinician are necessary.

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Introduction

Mastitis is defined as mammary gland inflammation which has infectious and non-infectious etiologies. Traditionally mastitis is classified into three categories including clinical, subclinical and chronic. Clinical mastitis is characterized by visible signs such as swollen udders which is felt hot in touching and changes in the physical appearance and taste of milk. Clinical mastitis may develop into systemic disease and endanger animal's life. In contrast, there are no visible changes in subclinical and chronic mastitis; therefore, somatic cell count and microbiological examination are essential for the diagnosis of animals affected by these types of mastitis (1).

Mastitis causes great economic concerns in domestic animals raised for milk production. Recently, raising dairy goats has taken into consideration in Iran since compare with cattle, they have more efficient digestive system, smaller body size and lower food intake which all make them cheaper source of protein. Highly milk productive goats are able to produce milk as much as 20 times their body weight (2).

Saanen is one of the most milk-producing goat breed worldwide. In the best condition, the average milk yield of a Saanen goat, can be 10 times that of other goats (3). Alpine is another milk-producing breed but with less average milk yielding compared to Saanen (2). As the main purpose of commercial Saanen and Alpine farms is milk production, regular monitoring of mastitis pathogens is essential.

Caprine mastitis occurs by a number of pathogens particularly bacteria and the most important of which is *Staphylococcus*. Staphylococcal mastitis usually is divided into *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS). CoNS are more prevalent and may lead to a persistent infection which effects on somatic cell count (1). *Streptococcus*, and coliforms are the other major causes of mastitis (4). The bacterial pathogens which are more involved

in outbreaks of clinical mastitis are *S. aureus*, *Streptococcus* spp. (*S. uberis*, *S. agalactiae*, and *S. suis*), and opportunists such as *Aspergillus*, *Pseudomonas*, *Burkholderia*, and *Serratia* (5, 6). Some of the infectious mastitis has systemic consequences in animals. It has been shown that 32% of mastitis cases caused by coliform are associated with bacteremia (7, 8).

Antibiotic resistance among mastitis pathogens has been increased by long-time usage of antibiotics and incomplete treatment which is common among farmers (9). Antimicrobial resistance of *S. aureus* and *E. coli* tetracycline has been reported recently (10, 11). This antibiotic is using frequently by farmers and its consequences would be appeared in future.

The purpose of the present study was to isolate the udder pathogenic bacteria from clinically infected Saanen and Alpine goats and determination of the antibiotic resistance profile in their milk samples.

Materials and Methods

Study setting and sample collection

During 2017-2019, milk sample of 55 goats including 26 Saanen and 29 Alpine breeds with clinical mastitis was aseptically collected into sterile tubes (6). The samples were placed next to ice and transferred to the laboratory for bacteriological analysis.

Bacterial isolation and identification

Fifty-five samples were initially streaked on general and differential culture media including blood agar, MacConkey agar and Eosin Methylene Blue (EMB). After incubation at 37°C for 24 hours, the grown colonies were morphologically examined and the colonies smears were stained by Gram staining method. According to the microscopy observations, the colonies were assessed by biochemical tests. In order to detect the

infection of the collected milk samples with *Trueperella pyogenes*, *Corynebacterium pseudotuberculosis*, *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus agalactiae*, the suspected colonies the following tests were performed: catalase, gelatinase, nitrate reduction, litmus milk, CAMP, coagulase and IMViC. Meanwhile, the colonies were inoculated into media including urea, loeffler serum, TSI and SIM. The colonies ability to ferment glucose, lactose, mannitol, and sucrose was also assessed by inoculating into liquid sugar media.

DNA extraction and Polymerase chain reaction

DNA was extracted from the milk samples using CinnaGen DNA extraction kit. Then PCR was performed for the detection of *Mycoplasma* spp. according to the method of Botes et al. in 2005 (12).

The primers amplified a 280-bp fragment of *Mycoplasma* 16S rRNA which was specific for the genus. The sequences included GPO3F 5'-TGGGGAGCAAACAGGATTAGATACC-3' and MGSO 5'-TGCACCATCTGTCACCTCTGTT AACCTC-3', respectively.

PCR was performed using 2 µM of each primer, 12.5 µl of 10X PCR master mix, 6 µl of DNA template and 2.5 µl of nuclease free water. The final volume of reaction mixture was amounted to 25 µl. Amplification was carried out in the automated DNA thermal cycle using the following cycling parameters: Initial denaturation at 94 °C for 2min, subsequently 35 cycles of denaturation at 94 °C for 15s, 59.3°C for 15s and 72°C for 15s. The final extension was performed at 72 °C for 5 min. The amplified products were observed in a one percent agarose by electrophoresis with 10 µl safe stain and Tris Borate EDTA buffer as the matrix. Then the products were visualized under a UV transilluminator.

Antimicrobial Susceptibility Testing

After isolation of the bacteria, a loop of each colony was added in the sterilized physiology serum and the turbidity was adjusted to 0.5 McFarland density standard. Using a sterile swab, the suspension was cultured on Mueller–Hinton agar. The cultured inoculums were tested for antimicrobial susceptibility to 12 antibiotic agents including penicillin (10 U), tylosin (30 µg), florfenicol (30 µg), tulathromycin (30 µg), enrofloxacin (5 µg), ceftiofur (30 µg), oxytetracycline (30 µg), lincomycin (15µg), streptomycin (10µg), ceftriaxone (30 µg), gentamicin (10µg) and amoxicillin (10µg). Then the plates were incubated at 37°C for 24 hours. The diameter of inhibition zone around each disk was measured and the results were reported as susceptible (S), intermediate (I), or resistant (R) according to the Clinical Laboratories Standards Institute guidelines (13).

Statistical analysis

Data obtained from SPSS software (version 24) at the level of $p < 0.05$ were analyzed using Mann-Whitney test and multivariate logistic regression model to assess the significant difference between drug resistance levels in Saanen and Alpine breeds.

Results

Mycoplasma spp. DNA was observed in 12.73% of the milks. *Escherichia coli* frequently most frequent isolated pathogen (29.1%). The Alpine breed showed a significantly different drug resistance pattern compare to the Saanens (Tables 1 and 2).

Molecular results

DNA of *Mycoplasma* spp. (Fig. 1) was detected in 7 out of 55 milk samples (12.73%) (Table 1).

Microbial culture

The frequency of isolated bacteria included the following respectively: *E. coli* (29.1%), *T. pyogenes* (25.5%), *S. aureus* (16.4%), *S. agalactiae* (9.1%), CoNS (5.5%), and *C. pseudotuberculosis* (1.8%) (Table 1).

Antimicrobial resistance

Results of antibiotic resistance are presented in Table 2. According to the antimicrobial susceptibility testing, *E. coli* had the most resistance to tylosin, florfenicol and streptomycin, but it was more susceptible to penicillin, gentamicin and tulathromycin respectively.

S. agalactiae had the highest resistance to lincomycin, oxytetracycline and ceftriaxone, but it was more susceptible to florfenicol and penicillin respectively. *S. aureus* showed the highest resistance to lincomycin and streptomycin, but it was more susceptible to amoxicillin and florfenicol with 66.7% difference compared to the other antibiotics.

Coagulase negative staphylococci showed the highest resistance to penicillin, gentamicin, lincomycin, and streptomycin, but they were more susceptible to florfenicol, enrofloxacin, amoxicillin and oxytetracycline with 33.3% difference compared to the other antibiotics.

T. pyogenes had 85% resistance against streptomycin, oxytetracycline and ceftriaxone, but it showed more susceptibility to gentamicin, amoxicillin, tulathromycin and ceftiofur respectively.

C. pseudotuberculosis showed the highest resistance to penicillin, florfenicol, enrofloxacin, amoxicillin, oxytetracycline and streptomycin, but it was more susceptible to gentamicin, ceftriaxone and ceftiofur respectively.

The significant difference between Saanen and Alpine was merely observed in antibiotic resistance to amoxicillin (0.039) which showed higher resistance in Alpine breeds (Table 2).

Discussion

The results of the current study indicated the interference of both environmental and contagious agents in goat's mastitis and *E. coli* as an environmental bacterium was the most frequent isolated pathogen in the milk samples (29.1%). Bradley and Green (2001), reported *E. coli* as the predominant pathogen isolated from the six studied herds in Somerset in all the months of the year (14).

The other isolated bacteria in the present study were *T. pyogenes*, *S. aureus*, *Mycoplasma* spp., *S. agalactiae*, CoNS and *C. pseudotuberculosis* respectively. In line with the current results, several studies have also shown that the most important pathogens of mastitis are *Staphylococcus*, *Streptococcus*, *Mannheimia haemolytica*, *Mycoplasma agalactiae*, *Trueperella pyogenes*, and coliforms (15, 16, 17, 18).

Zhao et al. (2014) reported that bacterial agents such as *Staphylococcus* spp., *Streptococcus* spp., and *E. coli* are the main causative organisms of mastitis in goats (16). In the study of Goncagul et al. (2021) in Turkey, non-aureus staphylococci and *E. coli* were respectively the most frequent bacteria in subclinical mastitis cases of Saanen breeds (18.9%). In addition, the authors isolated *S. aureus*, *S. agalactiae*, and *S. epidermidis* from apparently healthy goats (17).

Mastitis is a multifactorial disease which is affected by factors such as farm's sanitary condition, environmental condition and animal status (19). Isolation of various pathogens from goat herds is strongly associated with the unsuitable herd's hygiene status and incompetence management system (20). Moreover, the prevalence of contagious and environmental bacteria which we were isolated with high percentage in the current study, is associated with contaminated bed with feces and urine (21). Isolation of different pathogens should be considered as a serious warning in industrial farms. Therefore, as eradication is not rational, implementation of biosecurity plans, hygiene and

proper disinfection are the most cost-effective ways to control mastitis (21).

Our study evaluated the frequency of mastitis bacteria in Saanen and Alpine breeds for the first time in Iran and meanwhile antibiotic resistance of each bacterium was assessed in both of the breeds. According to the results, the difference in antibiotic resistance between Saanen and Alpine was merely seen in the case of amoxicillin (Table 2). Alpine goats showed significantly higher resistance to amoxicillin compare to the Saanens (p value=0.037). *C. pseudotuberculosis* isolates were resistant to amoxicillin. In contrast, CoNS and *S. aureus* were completely susceptible to amoxicillin and florfenicol. Jabbar et al. (2020) in Pakistan found the same results and they considered amoxicillin as the choice drug for *Staphylococcus* (22). In the study of Balemi et al. (2021) in Ethiopia, *S. aureus* was the most prevalent agent among other bacteria in goats and it was 100% resistant to penicillin G and spectinomycin, while it showed 100% susceptibility to doxycycline, ceftriaxone, and vancomycin (19).

In the present study, *S. aureus* was highly resistant to streptomycin and lincomycin (100%), but in case of CoNS, 100% resistance was seen to penicillin, gentamicin, lincomycin and streptomycin.

In the study of Goncagul et al. (2021), *E. coli* was 100% susceptible to amoxicillin (17), while our investigation identified penicillin, tulathromycin and gentamicin as the antibiotic choices for *E. coli*. Furthermore, an in vitro study in 2010 showed that generally gentamicin is more effective against mastitis bacteria (23).

Contrary to the current evaluation, in the study of Begum et al. (2016) tetracycline was identified to have the highest efficacy against mastitis bacteria in goats (24). We identified oxytetracycline as one of those antibiotics which *S. agalactiae*, *T. pyogenes* and *C. pseudotuberculosis* showed the highest resistance to. A similar observation was made by Ramprabhu (2019) who found the isolated bacteria from subclinical mastitis milk of goats

were relatively resistant to tetracycline and amoxicillin. The researcher claimed it might be the consequence of the variation in the usage levels of these antibiotics (25). Excessive administration and lack of supervision over oxytetracycline usage by farmers in small ruminants are important causes of increasing bacterial resistance to this antibiotic in Iran.

Antibiotics are routinely used in the treatment of mastitis. However, the more occurrences of mastitis happen in a farm, the more unconscious use of antibiotics in infected animals takes place. It eventually leads to an increase in the risk of antibiotic resistance which the presence of them in milk particularly *S. aureus* is hazardous for public health (26). Therefore, precise diagnosis and identification of the pathogens causing mastitis and the use of effective antibiotics are necessary to determine treatment strategies especially in the future cases of mastitis.

Based on the obtained data and high multidrug resistance of the microorganisms, using antibiogram by clinician is necessary in the monitoring of the bacterial resistance in a flock. Avoidance from applying numerous and unnecessary antibiotics, especially since mastitis is one of the recurring causes of removal of animals from the farm, is another important proceeding.

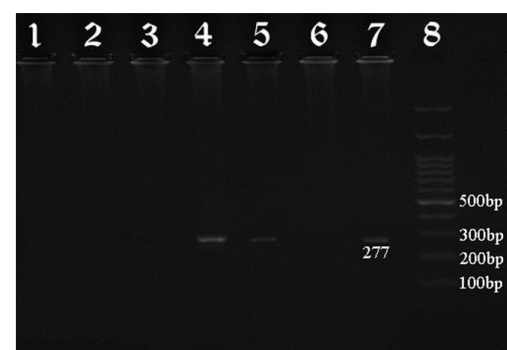


Fig 1. Amplification fragments of conventional PCR. 8: 100-bp ladder; 7: Positive control; 6: negative control; 5, 4: *Mycoplasma*-positive samples.

Table 1. Frequency of isolated bacteria from the milk samples.

Isolated bacteria	Frequency	Percentage	Cumulative percentage
<i>E.coli</i>	16	29.1	29.1
<i>T.pyogenes</i>	14	25.5	80
<i>S.aureus</i>	9	16.4	45.5
<i>S.agalactiae</i>	5	9.1	54.5
CoNS	3	5.5	100
<i>C. pseudotuberculosis</i>	1	1.8	81.8
<i>M. agalactiae</i>	7	12.7	94.5
Total	55	100	

Table 2. Antibiotic resistance in Saanen and Alpine breeds. Pvalue<0.05 was considered as statistically significant.

Antibiotic	Breed	Antimicrobial resistance				P-value
		S*	I*	R*	r*	
Tulathromycin	Saanen	7	0	3	13	0.852
	Alpine	9	0	2	14	
Ceftiofore	Saanen	11	4	1	7	0.388
	Alpine	7	3	5	10	
Tylosin	Saanen	0	3	4	16	0.674
	Alpine	3	2	0	20	
Penicillin	Saanen	12	1	10	0	0.541
	Alpine	9	5	11	0	
Gentamicin	Saanen	13	4	3	3	0.614
	Alpine	12	5	5	3	
Florfenicol	Saanen	11	0	0	12	0.161
	Alpine	7	0	0	18	
enrofloxacin	Saanen	4	1	10	8	0.637
	Alpine	4	1	14	6	
Amoxicillin	Saanen	12	2	1	8	0.037**
	Alpine	6	0	4	15	
Ceftriaxone	Saanen	5	3	6	9	0.174
	Alpine	2	3	9	11	
Oxytetracycline	Saanen	3	0	8	12	0.773
	Alpine	2	1	8	14	
Streptomycine	Saanen	1	0	2	20	0.626
	Alpine	2	0	0	23	
Lincomycin	Saanen	0	0	0	23	0.170
	Alpine	0	2	0	23	

In addition, after determination of bacterial susceptibility to antibiotics, the results of a specific antibiotic usage should be monitored.

Conclusion

In conclusion, sampling of a larger range of animals and identification of subclinical cases, faster diagnosis and treatment of the disease which prevents mastitis progression and further economic damages are strictly recommended. We showed higher bacterial resistance to amoxicillin in the samples from Alpine breeds, so further studies are needed to evaluate the issue of antibiotic resistance in this breed.

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Ethics approval and consent to participate

This study did not require an ethics license.

Conflict of interest

The authors declare that they have no conflict of interest.

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