The frequency of bacterial pathogens of mastitis and their antibiotic susceptibility in Saanen and Alpine goats

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Research Article

Keywords: Alpine, Antibiotic resistance, Goat, Mastitis, Saanen

Posted Date: November 8th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-845604/v1

Abstract

Mastitis is mammary gland inflammation and as the purpose of Saanen and Alpine farms is milk production, control of mastitis is important. Antimicrobial resistance among mastitis pathogens has gradually been increasing. The aim of the present study was to isolate pathogenic bacteria from mastitis cases in Saanen and Alpine goats and to determine their antibiotic resistance in milk. The milk sample of 26 Saanen and 29 Alpine breeds with clinical mastitis was collected and cultured on general microbiological media. Then, the colonies were stained by gram staining and were assessed by differential media and biochemical tests. PCR was performed for the detection of *Mycoplasma* spp. The isolated bacteria were tested against 12 antibacterial disks. The significant difference in drug resistance levels between the Saanen and Alpine breeds was statistically assessed. *Mycoplasma* spp. was detected in 12.73% of samples. The frequency of the isolated bacteria was *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%) respectively. The significant difference between Saanen and Alpine was observed in antibiotic resistance to amoxicillin which showed more resistance in the Alpine breed. All the isolated bacteria showed multidrug resistance. Based on the obtained data using of antibiotics more accurate and using antibiogram test by clinician is necessary in the treatment of mastitis.

Introduction:

Mastitis is defined as mammary gland inflammation which has infectious and non-infectious etiologies. Traditionally mastitis is divided into three categories including clinical, subclinical and chronic. Clinical mastitis is characterized by visible symptoms such as swollen udders which is felt hot in touching and changes in the appearance and taste of milk. Clinical mastitis may lead to systemic disease and endanger animal's life. In contrast, there are no organoleptic changes in subclinical and chronic mastitis; therefore, diagnosis needs somatic cell count and microbiology confirmatory tests (Bachaya et al., 2005).

Mastitis causes great economical concerns in domestic animals raised for milk production. Goats are beneficial animals and their milk, skin, hair and in some breeds wools are utilizable throughout the world. Highly milk productive goats are able to produce milk as much as 20 times their body weight (Gökdai *et al*, 2020). Recently raising of dairy goats has taken into consideration in Iran since compare with cattle, dairy goats have more efficient digestive system, smaller body size and lower food intake which all make them cheaper source of protein.

Saanen is one of the most milk-producing goat breed worldwide. In the best condition, the average milk yield of a Saanen goats, can be 10 times that of other hair goats (Görgülü, 2014). Alpine is another milk-producing breed but with less average milk yielding compared to Saanen (Gökdai *et al*., 2020). As the main purpose of Saanen and Alpine farms is milk production, control of mastitis pathogens should be monitored.

Caprine mastitis occurs by a number of pathogens particularly bacteria (Bradley, 2002) which the most important ones is *Staphylococcus* (El-jakee *et al*., 2013). 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 (El-jakee *et al*., 2013). *Streptococcus*, and coliforms are the other major causes of mastitis (Keane, 2019). 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* (Croft et al. 2000; Plummer and Plummer 2011). Some of the infectious mastitis has systemic consequences in animals. Several studies have shown that 32% of mastitis cases caused by coliform are associated with bacteremia (Cebra et al., 1996; Wenz et al., 2001).

It is evident that there is some antimicrobial resistance among mastitis pathogens which has been increased by long-time usage of antibiotics and incomplete treatment which is common among farmers (Memon et al., 2013). Antimicrobial resistance of *S.aureus* and *E.coli* against tetracycline is recently approved (Abdi et al., 2018; Massé et al., 2021). Bacteria which are resistant to more than two antibiotics are considered as multiple drug resistant (MDR) and using the infected milk may transfer the resistance genome to normal felora of consumers (Najeeb et al., 2013). This issue makes treatment protocols inefficient and has public health hazard.

The purpose of the present study was to isolate the udder pathogenic bacteria from clinically infected Saanen and Alpine goats and determination of their 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. The samples were placed next to ice and transferred to the laboratory.

Bacterial isolation and identification:

The milk samples were initially streaked on general microbiological media including blood agar, MacConkey agar and Eosin Methylene Blue (EMB) and then, were incubated at 37°C for 24 hours. The colonies which were grown in the media were morphologically examined and stained by gram staining method. According to the bacteria observed under light microscope, the colonies were assessed by differential media and biochemical tests. In order to examine the infection of the mastitis milk with *Trueperella pyogenes*, *Corynebacterium pseudotuberculosis*, *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus agalactiae* the suspected colonies were used for complimentary tests including catalase, gelatinase, nitrate reduction, litmus milk, CAMP, coagulase and IMViC. Meanwhile, the colonies were inoculated into complimentary media including urea, loeffler serum, TSI and SIM. The colonies were also inoculated into sugar liquid media to identify their ability of fermentation of glucose, lactose, mannitol, and sucrose.

Polymerase chain reaction:

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

The primers amplified a 280-bp fragment of *Mycoplasma* 16S rRNA which were specific for the genus and included GPO3F 5'-TGGGGAGCAAACAGGATTAGATACC-3' and MGSO 5'-TGCACCATCTGTCACTCTGTTAAC CTC-3' respectively.

PCR was performed using 2 µM of each primer, 12.5 µl 10X PCR master mix, 6 µl template DNA and 2.5 µl nuclease free water. The final volume of reaction mixture was 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 colonies was added in the sterilized physiology serum to reach to the 0.5 McFarland density standard. Using a sterile swab, the suspension was cultured on muellerhinton agar. The cultured inoculums were tested against 12 antibacterial disks 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 kept 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 (Wayne, 2018).

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 the Saanen and Alpine breeds.

Results:

Molecular results:

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

Table 1
Frequency of the isolated bacteria from the milk samples

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

Microbial Culture:

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

Antimicrobial Resistance:

There was a distinct difference in antimicrobial resistance among the isolated bacteria between Saanen and Alpine goats (Table 2). However, the significant difference between Saanen and Alpine was merely observed in antibiotic resistance to amoxicillin (P = 0.037) which showed more resistance in the Alpine breed (Table 2).

Table 2
Antibiotic resistance in saanen and alpine breeds and pvalue < 0.05 was considered as statistically significant

Antibiotic	Breed	Antimicrobial resistance				P-value
		S*	I*	R*	r*	0.852
Tulathromycin	Saanen	7	0	3	13	
	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	

*S: sensitive, I: intermediate, R: resistant, r: resistance without inhibition zone around.**Significant difference

According to the results of antimicrobial susceptibility testing, *E.coli* had the most resistance against tylosin, felorfenicol and streptomycine, but it was more sensitive against penicillin, gentamicin and tulathromycin respectively.

S. agalactiae had the most resistance against lincomycin, oxytetracycline and ceftriaxone, but it was more sensitive against felorfenicol and penicillin respectively. *S. aureus* showed the most resistance against lincomycin and streptomycine, but it was more sensitive against amoxicillin and felorfenicolwith 66.7% difference compared to the other antibiotics.

Coagulase negative staphylococci had the most resistance against penicillin, gentamicin, lincomycin, and streptomycine but they were more sensitive against felorfenicol, enrofloxacin, amoxicillin and oxytetracycline with 33.3% difference compared to the other antibiotics.

T.pyogenes had 85% resistance against streptomycine, oxytetracyclineand ceftriaxone, but it was more sensitive against gentamicin, amoxicillin, tulathromycin and ceftiofore respectively.

C. *pseudotuberculosis*had the most resistance against penicillin, florfenicol, enrofloxacin, amoxicillin, oxytetracycline and streptomycine, but it was more sensitive against gentamicin, ceftriaxone and ceftiofore respectively.

Discussion:

The results of the current study indicated the interference of both environmental and contagious agents in the 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 (Bardley and Green, 2001).

The other bacteria were detected in the present study included *T.pyogenes*, *S.aureus*, *Mycoplasme spp.*, *S. agalactiae*, CoNS and *C. pseudotuberculosis* respectively. Generally, the most important pathogens of mastitis are *Staphylococcus*, *Streptococcus*, *Mannheimia haemolytica*, *Mycoplasma agalactiae*, *Trueperella pyogenes*, and coliforms (Esmaeili and Hamedi, 2019).

It has been approved that bacterial agents such as *Staphylococcus* spp., *Streptococcus* spp., and *E. coli* are the main causative organisms of mastitis in goats (Zhao et al., 2014). In the study of Goncagul et al (2021) in Turkey, non-aureus staphylococci and *E.coli* were respectively the most frequent bacteria in mastitis cases in Saanen breed (18.9%). The authors found *S. aureus*, *S. agalactiae*, and *S. epidermidis* as the other isolated bacteria from apparently healthy goats (Goncagul et al., 2021).

Mastitis is a multifactorial diseas in which the farm's sanitary condition, environmental factors and animal status are involved (Balemi et al., 2021). It is evident that isolation of various pathogens from goat herds is strongly associated with the unsuitable herd's hygiene status and its incompetence management system (McDougall et al., 2002). Moreover, the prevalence of contagious and environmental

pathogens which we have isolated with high percentage, is associated with contaminated bed with faces and urine (Radostis *et al*., 2007). 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.

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 bacteria was assessed in both breeds. According to the results, the difference in antibiotic resistance between Saanen and Alpine was merely seen in the case of amoxicillin (Table 2). The Alpine goats were significantly more resistance to amoxicillin compare to the Saanens. Resistance to amoxicillin was seen in *C.pseudotuberculosis*, but amoxicillin and florfenicol were the best choices against CoNS and *S. aureus*. Jabbar et al. (2020) in Pakistan found the same results and they considered amoxicillin as the choice drug against *Staphylococcus* (Jabbar et al., 2020). 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 spectinomycine, but it showed 100% sensitivity to doxycycline, ceftriaxon, and vancomycine (Balemi et al., 2021).

By contrast *S. aureus* sensitivity to amoxicillin in the current study, this bacterium was highly resistant to streptomycin and lincomycin (100%), but in case of CoNS, 100% resistance was seen towards penicillin, gentamicin, lincomycin and streptomycin. In the study of Goncagul et al. (2021), *E.coli* was 100% sensitive to amoxicillin (Goncagul et al., 2021), while our investigation identified penicillin, gentamicin and tulathromycin as the antibiotic choices for *E. coli*. Furthermore, an in vitro study in 2010 showed that generally gentamicin is more effective against mastitis bacteria (Ali et al., 2010).

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 (Begum et al., 2016). We identified oxytetracycline as one of those antibiotics which *S. agalactiae*, *T. pyogenes* and *C. pseudotuberculosis* showed the most resistance against. Similarly, Ramprabhu (2019) found the isolated bacteria from subclinical mastitis milk of goats are relatively resistant to tetracycline and amoxicillin. The researcher claimed that it might be as a result of variation in the level of usage of these drugs (Ramprabhu, 2019). Excessive administration and lack of supervision over oxytetracycline usage by farmers in small ruminants is one of the 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.auerus* is hazardous for public health (Rainard et al., 2018). Therefore, precise diagnosis and identification of the etiological agents causing mastitis and the use of effective antibiotics are necessary to determine treatment strategies.

Based on the obtained data and high multidrug resistance of the microorganisms, using antibiogram test by clinician is necessary in the treatment of mastitis. 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. In addition, after determination of bacterial susceptibility against antibiotics, the results of the usage of specific antibiotics should be monitored. 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 significantly higher resistance to amoxicillin in Alpine breed so further studies are needed to evaluate the issue of antibiotic resistance in this breed.

Declarations

Acknowledgement

The authors acknowledge the members of microbiology laboratory of the faculty of veterinary medicine, University of Tehran.

Code availability: All codes analyzed during this study are included in this published article.

Funding: This project was supported by faculty of veterinary medicine, University of Tehran.

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

Code availability: Codes used for the statistical analysis and tables creation are available on request through the corresponding author.

Authors 'contributions

Hossein Esmaeili: Investigation, Conceptualization, Formal analysis, Resources, Writing - Review & Editing Supervision, Project administration, Resources. *Parviz Tajik:* Investigation, Validation, Resources, Conceptualization.

Mona Hamedi: Investigation, Methodology, Data curation, Supervision.

Amir pasha Shakeri: Investigation, Methodology, Data curation.

Ethics approval: The manuscript does not contain clinical studies or patient data.

Consent to participate: All the authors approved the final manuscript.

Consent to publication: All the authors consented the final manuscript.

Conflict of Interest: The authors declare no competing interests.

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