Investigation of Virulence Properties of *Staphylococcus* Species Isolated from Goats with Subclinical Mastitis

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ABSTRACT

Identification of coagulase-negative staphylococci (CNS) is tedious and sometimes cannot be done satisfactorily since many biochemical tests are required in routine diagnostic laboratories. CNS, which play an important role in the aetiology of mastitis, are important members of the skin and mucous membranes and cause infections especially in immunocompromised individuals. The increasing incidence of CNS, especially in the aetiology of mastitis, requires the correct identification of *Staphylococci* isolates at the species level. In recent years, *tuf* gene analysis has been frequently preferred for species identification of CNS due to its practicality and reliable results. The aim of this study was to identify *Staphylococcus* species by conventional and molecular methods from Saanen goat milk with mastitis and to determine the virulence genes and the antibiotic susceptibility of the isolates. In this study, 41 (37.2%) Gram-positive cocci were isolated from the 110 goat milk samples with subclinical mastitis. Bacterial identification was carried out using conventional methods. All Gram-positive cocci were examined with the BD Phoenix™ 50 device for identification and antibiogram susceptibility analyses. Polymerase chain reaction (PCR) was used to confirm the species level identification of the isolates, and to identify virulence genes. After the identification of the *Staphylococcus* isolates, analysis for the presence of 16S rRNA, *nuc*, *tuf*, *coa* genes, virulence genes, enterotoxin genes were performed for genotypic verification by PCR. The results were as follows: 5 (12.2%) *S. pettenkoferi*; 5 (12.2%) *S. aureus*; 4 (9.7%) *S. epidermidis*; 3 (7.3%) *S. equorum*; 3 (7.3%) *S. warneri*; 3 (7.3%) *S. caprae*; 2 (4.9%) *S. capitis*; 1 (2.4%) *S. simulans* and 15 (36.6%) *Streptococcus* spp. were identified using BD Phoenix™. *tuf* gene sequencing results of *Staphylococcus* isolates (n=26) showed that; 5 (19.2%) *S. aureus*, 4 (15.4%) *S. caprae*, 4 (15.4%) *S. succinus*, 4 (15.4%) *S. devriesei*, 3 (11.5%) *S. chromogenes*, 1 (3.8%) *S. heamolyticus*, 1 (3.8%) *S. petracii sp. jettensis*. The resistance to antimicrobial drugs ampicillin, penicillin and amoxicillin-clavulanic acid was 88.4%, 88.4%, 84.4%, respectively. The prevalence of the studied virulence genes was *sea* 3.8%, *seb* 30.7%, *sec* 11.5%, PVL 3.84%, *Hla* 53.8%, *Hlb* 34.6%, and *BAP* 7.7%. Our results confirmed the predominance of *S. caprae* and *S. succinus* among CNS isolates. *Tuf* genes are considered as an accurate tool for the identification of CNS strains. Finally, the use of advanced diagnostic methods in goat mastitis cases and the determination of antibiotic susceptibility of *Staphylococcus* isolates will preclude the occurrence of disease that may harm public health.

Keywords: Mastitis, *Staphylococcus* spp.; Sequencing; Virulence Genes; Antibiogram.

INTRODUCTION

The *Staphylococcus* spp., *Streptococcus* spp., *Enterobacteriaceae* and *Mycoplasma* spp. are the most widespread pathogens that cause mastitis in goats. *Staphylococcus aureus* and coagulase negative *Staphylococcus* (CNS) play an important role in goat mastitis (1). Many methods have been developed for the identification of *Staphylococcus* species. Staphylococcal species can be rapidly identified using a variety of systems

based on biochemical reactions (i.e., tube tests, miniature biochemical tests, and computerized identification systems) (2). Several polymerase chain reactions (PCR), sequencing-based methods, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), the macrorestriction analysis of DNA-fragments (REA-PFGE), whole genome sequencing (WGS) enables rapid identification of organisms through molecular analysis which have been developed for the identification of staphylococci (3).

S. aureus, the major pathogen of the *Staphylococcus* genus, causes a wide variety of clinical infections in humans and animals (4). The distinction between *S. aureus* from most staphylococci is primarily made by evaluating its ability to produce the enzyme coagulase, which promotes coagulation of plasma. This activity is the basis of routine identification testing and aids in the distinction between *S. aureus* and CNS, long considered harmless but emerging as important opportunistic pathogens (5). The *coa* (coagulase) gene in *Staphylococcus* results in a secretory protein that causes blood coagulation through the activation of prothrombin inorder to overcome the host defence system (6). It is one of the major virulence proteins used as a basis for differentiation of coagulase positive staphylococci (CPS) from coagulase negative staphylococci (CNS) species. The nuclease (*nuc*) gene sequence is responsible for the thermonuclease activity specific for *Staphylococcus aureus*. The enzyme thermonuclease (TNase), which has exo- and endonuclease activity, cleaves DNA and RNA into nucleotides by hydrolysing the phosphodiester bonds in their structure (7).

The elongation factor Tu (*tuf*) gene sequence plays a role in peptide chain formation in *Staphlyococcus* species. The *tuf* gene cluster, which is located in the short tandem repeat region on the bacterial chromosome, shows a significant diversity among members of staphylococci. Its small size and its conserved location in bacterial chromosome enhances its superiority in DNA sequencing compared with 16S rRNA for construction of phylogenetic trees on species and genus level in staphylococci, enterococci, and streptococci isolates. It has been recognized that the *tuf* gene is a more specific gene than the 16S rRNA gene in the identification of CNS species (8).

Staphylococcus exotoxins play a role in mastitis infections and are consequently found in dairy products. Staphylococcal food poisoning (SFP) outbreaks occur as a result of human consumption of these foodstuffs. Rapid, sensitive and accurate detection of exotoxins in the mammary glands and indeed in food is crucial both to determine the best treatments for *S. aureus* infections and to prevent economic loss in the dairy industry (9).

S. aureus enterotoxins include toxic shock syndrome toxin 1 (TSST 1) and staphylococcal enterotoxins (SEs). Hemolysins attack cell membranes as β, α and δ toxins and cause platelet damage, lysosome destruction and necrosis. *Hla* (α hemolysin) is a toxin that disrupts the cell membrane, causes irreversible osmotic changes and causes cell death through apoptosis. *Hla* can damage the membranes of a variety of cells, such as lymphocytes, red blood cells, platelets, and endothelial cells (10). Beta hemolysin (*Hlb*) is encoded by a lysogenic bacteriophage and in itself it cannot destroy most cell types, but exposes sensitive cells to other lytic proteins such as *Hla* and leukocidins (11). Also known as sphingomyelinase, this toxin can also damage keratinocytes, helping bacteria to colonize mammalian skin. The different susceptibility of different types of erythrocytes to *Hlb* may be due to the amount of sphingomyelin present in the cells (10). Leucocidins are also pore-forming bicomponent toxins that attack immune cells. There are seven different types of leukocytes, and Panton-Valentine leukocidin (PVL) is the toxin with the strongest effect on immune cells (12).

To survive, bacteria have developed, various strategies such as development of biofilms to colonize and cause infections. Biofilm production is an important virulence factor. Biofilm-producing isolates can remain in the milk environment for a long period of time, transfer genetic material to other bacteria. Biofilm-associated protein (*BAP*) is a cell wall-bound surface protein encoded by the *BAP* gene, increasing the dispersal of virulence factors (13).

The aim of this study is to determine the important *Staphylococcus* species that play a role in the aetiology of goat mastitis using molecular methods, and to investigate the important virulence genes (enterotoxin genes, hemolysin genes, TSST, *BAP*, PVL) and antibiotic susceptibility of the isolates.

MATERIALS AND METHODS

Bacterial isolates

Routine mastitis screening was performed in 500 Turkish Saanen goats from five different farms in Aydın region in Turkey, in order to identify goats with subclinical mastitis.

Turkish Saanen goat is known for its high milk and reproductive efficiency in our country and has a widespread breeding area. Milk samples were taken by a veterinary practitioner. First of all, goats with clinical and subclinical mastitis were identified as described below:

Clinical Mastitis: Clinical findings like abnormalities of secretions, abnormalities of size, consistency and temperature of mammary gland were examined by visual inspection and palpation. Pain reaction upon palpation, changes in the milk (blood tinged milk, watery secretions, clots, pus), and changes in consistency of udder were considered as indications of the presence of clinical mastitis.

Subclinical Mastitis: Many tests to detect changes in milk with mastitis have been developed (14). California Mastitis Test (CMT) is one of these tests. As in other types of mastitis, the culture method is accepted as the "gold standard" in the diagnosis of subclinical mastitis. Goats, which did not have clinical mastitis, were subjected to further investigation for subclinical mastitis by using California Mastitis Test (CMT). The CMT was performed according to the procedure as previously described (14). The result was scored as 0, +1, +2 or +3 depending on the intensity of reaction. CMT result for goat milk samples accepted for score of 0 as negative and score of +1, +2, +3 and up as positive.

When taking samples, the teats were cleaned with 70% alcohol and the first 2 milkings were milked in another container. Then, the sampling was completed as 10 ml of milk sample into sterile tubes. The animals from which the samples were taken consisted of hair goats aged 2-5 years, which did not receive antimicrobial treatment during the milking period and in the last 1 month, had at least one birth. The CMT was examined according to the procedure as previously desvribed (14). The result was scored as 0, +1, +2 or +3 depending on the intensity of reaction. CMT result for goat milk samples accepted for score of 0 as negative and score of +1, +2, +3 and up as positive. A milk sample was taken from the mammary lobe with the highest CMT score. On hundred and ten milk samples with subclinical mastitis were collected from five farms. While collecting milk samples, care was taken that the animals had not received antibiotic treatment over the the last two weeks.

Isolation and identification

Milk samples were cultured in blood agar (Merck, Germany) at 37°C for 24 hours. Pure cultures were stained by the Gram staining method. Catalase test was applied to Gram positive cocci and strains that were Gram positive were subjected to the coagulase test. For the coagulase test, the isolates were recorded as either coagulase positive or coagulase negative. These isolates were passaged on Tryptic Soy Agar (TSA) (Merck, Germany) plates and incubated at 37°C for 18 hours. The identification and anti-biograms of the pure colonies formed after incubation in TSA were loaded into the BD Phoenix™ 50 automatic identification device (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). The biochemical identification data obtained from the device were evaluated.

DNA extraction

Total DNA extraction from isolates were performed as recommended by the manufacturer using the Thermo Scientific™ Genomic DNA Purification Extraction Kit (Waltham, Massachusetts, USA). The amount of DNA were measured with the Nanodrop device (Maestrogen®, Taiwan) and recorded. DNA purity and quantity controls were also performed. The ratio of OD260/OD280 was between 1.6-2.0 indicating that the DNA was pure (15). The extracted DNA samples were stored in cryotubes at -20°C.

PCR

PCR assays were performed to detect the presence of 16S rRNA gene (8), specific to *Staphylococcus* species and virulence genes; *nuc* (7), *coa* (6), *tuf* (8), PVL (17), TSST (18), *Hla* (17), *Hlb* (17), *BAP* (19) in all *Staphyococcus* spp. isolates (Table 1).

PCR, for each sample was carried out on a volume of 25 µl, final concentration was 10xTaq enzyme buffer solution 1x, 50 mM $MgCl_2$ 2 mM, 10 mM dNTP 0.2 mM, 100 ρmol primer (for each) 0.4 ρmol, 5 U Taq DNA polymerase 1.5 U (Fermentas, Massachusetts, USA), 3 µl of each DNA. The prepared tubes were loaded in the thermal cycler (Boeco, Hamburg, Germany). *S. aureus* ATCC 43300 was used as a positive control in PCR, and a DNA-free master mix was used as a negative control.

The DNA was amplified using the following protocol: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation (95°C for 30s), annealing for 30s [55°C (*Hla, Hlb,BAP,* PVL), 56°C (*tuf*), 57°C (TSST, *nuc,* 16S rRNA, *sea, seb, sec*), 58°C (*coa*)] and extension (72°C for 1 min), with a single final extension for 7 min at 72°C. On electrophoresis, a 1.5% agarose gel stained with Safe

Table 1. Primers used in this study.

	Genes	Sequence(5'/3')	Amplicon (bp)	Reference
Universal Primer	***16S* rRNA**	AGAGTTTGATCMTGGCTCAG CCGTCAATTCMTTTRAGTTT	527	8
***Staphylococcus* specific genes**	***coa***	ATAGAGATGCTGGTACAGG GCTTCCGATTGTTCGATGC	710	6
	nuc	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	279	7
	tuf	GCCAGTTGAGGACGTATTCT CCATTTCAGTACCTTCTGGTAA	412	8
Virulence factors	**PVL**	ATCATTAGGTAAAATGTCTGGACATGATCCA GCATCAASTGTATTGGATAGCAAAAGC	433	17
	TSST	ATGGCAGCATCAGCTTGATA TTTCCAATAACCACCCGTTT	350	18
	Hla	CTGATTACTATCCAAGAAATTCGATTG CTTTCCAGCCTACTTTTTTATCAGT	209	17
	Hlb	GTGCACTTACTGACAATAGTGC GTTGATGAGTAGCTACCTTCAGT	309	17
	BAP	CCCTATATCGAAAGGTGTAGAATTG GCTGTTGAAGTTAATACTGTACCTGC	971	19
Enterotoxin genes	***sea***	TTGGAAACGGTTAAAACGAA GAACCTTCCCATCAAAAACA	120	18
	seb	TCGCATCAAACTGACAAACG GCAGGTACTCTATAAGTGCC	478	18
	sec	GACATAAAAGCTAGGAATTT AAATCGGATTAACATTATCC	257	18

View (100 ml/6 µl) (ABM, Richmond, Canada) was used and the gel was exposed to 100 volts for 45 min. After electrophoresis, the gel was placed in the chamber of the transilluminator device, which was connected to the computer and photographed under UV light (Vilbert Lourmat, Collegien, France). When the amplified product formed a band of the expected size (Table 1.), it was assumed to carry the gene examined.

Sequence analysis of *Staphylococcus* isolates

Sequence analysis for the *tuf* gene was carried out. For this purpose, PCR products were first purified for sequence analysis. For purification, ExoSAP-IT™ (GML®) (PCR Product Cleanup Reagent, Waltham, Massachusetts, United States) was used. Sephadex (GML®) (Sigma-Aldrich, St. Louis, Missouri, United States) was used for the purification of the products obtained from sequence PCR.

Two microliters of ExoSAP was distributed to sterile PCR tubes and 5 µl of PCR product was added to purify the 16S RNA gene region obtained after *Staphylococcus* PCR, resulting in a total volume of 7 µl. Then the resulting mixture was loaded into the thermocycling device. Purified PCR products, Applied Biosystems™ BigDye™ Terminator, version 3.1. (Waltham, Massachusetts, United States) were prepared for sequencing PCR reactions. Sequencing was performed using the PCR products. The results obtained were compared electronically with the NCBI Blast® nucleotide sequences and the percent similarity rates were determined.

Determination of antimicrobial susceptibility

The antibiogram susceptibility test performed with PMIC/ID87 kit (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) into the BD Phoenix™ M50 automatic identification device with CLSI standards. PMIC/ID87 kit contained the following antibiotics: Amoxicillin-clavulanic acid, ampicillin, cefoxitin, ciprofloxacin 40, clindamycin, daptomycin, erythromycin, fosfomycin, fusidic acid, gen-

Table 2. *Staphylococcus* spp. MIC standard values and (n=26) *staphylococcus* isolates MIC values results (16).

Antibiotics		Antibiotic contents	*Staphylococcus* isolates	*Staphylococcus* isolates (n=26)	
		(µg/mL)	MIC RANGE	S(%)	R(%)
Amoxicillin/Clavulanate	AMC	2/1 – 8/4	≤0.5 – ≥32	4 (15.4)	22 (84.6)
Ampicillin	AM	2 – 8	≤4 – ≥128	3 (11.5)	23 (88.5)
Cefoxitin	FOX	2 – 8	≤0.25 – ≥4	26 (100.0)	0 (0.0)
Ciprofloxacin	CIP	1 – 4	≤0.125 – ≥1	26 (100.0)	0 (0.0)
Clindamycin	CC	0.25 – 1	≤0.125 – ≥8	21 (80.8)	1 (3.9)
Daptomycin	DAP	0.25 – 4	≤0.125 – ≥4	25 (96.1)	1 (3.9)
Erythromycin	E	0.25 – 2	≤0.125 – ≥4	25 (96.1)	0 (0.0)
Fosfomycin	FF	16 – 64	≤4 – ≥256	22 (84.6)	4 (15.4)
Fusidic Acid	FA	0.5 – 8	≤0.125 – ≥4	24 (92.3)	2 (7.7)
Gentamicin	GM	1 – 4	≤0.5 – ≥4	26 (100.0)	0 (0.0)
Levofloxacin	LVX	1 – 4	≤0.125 – ≥1	26 (100.0)	0 (0.0)
Linezolid	LZD	0.5 – 4	≤0.25 – ≥8	25 (96.1)	1 (3.9)
Nitrofurantoin	FM	16 – 64	≤2 – ≥64	24 (92.3)	2 (7.69)
Oxacillin	OX	0.25 – 2	≤0.25 – ≥16	20 (76.9)	6 (23.1)
Penicillin	P	0.0625 – 0.25	≤0.125 – ≥4	3 (11.5)	23 (88.5)
Quinupristin / Dalfopristin	SYN	0.5 – 2	≤0.125 – ≥16	21 (80.8)	1 (3.9)
Rifampin	RA	0.25 – 1	≤0.25 – ≥2	25 (96.1)	0 (0.0)
Teicoplanin	TEC	0.5 – 4	≤0.5 – ≥8	25 (96.1)	1 (3.9)
Tetracycline	TE	0.5 – 2	≤0.5 – ≥64	19 (73.1)	7 (26.9)
Tigecycline	TGC	0.25 – 1	≤0.25 – ≥4	25 (96.1)	1 (3.9)
Tobramycin	NN	1 – 4	≤0.25 – ≥4	26 (100.0)	0 (0.0)
Trimethoprim/Sulfamethoxazole	SXT	0.5/9.5 – 2/38	≤0.25 – ≥2	26 (100.0)	0 (0.0)
Vancomycin	VA	0.5 – 4	≤0.25 – ≥4	26 (100.0)	0 (0.0)

S (%) – Percentage senstitive
R (%) – Percentage resistant

tamicin, levofloxacin, linezolid, nitrofurantoin, oxacillin, quinopristin-daltomycin, tetracycline, tigecycline, tobramycin, trimethoprim-sulfamethoxazole, vancomycin. The minimum inhibitory concentration (MIC) standard values of these antibiotics are shown in the Table 2.

Statistical Analyses

Statistical analyses were performed by the commercial statistical software SPSS version 21.0 (SPSS Inc., USA). The Chi square test was used to compare the relationship between antibiotic susceptibility and resistance status in isolates carrying and not carrying the coagulase gene. The results were evaluated at a confidence interval of 95%. A P value <0.05 was considered as statistically significant.

RESULTS

Bacterial isolation

Forty-one (37.2%) Gram-positive cocci were isolated from the CMT positive 110 milk samples from cases of subclinical mastitis examined in the study. The catalase test performed on the 41 Gram positive isolates; 26 (63.4%) isolates were found to be catalase positive and the other 15 (36.6%) isolates found to be negative. Because of the conventional coagulase test performed on catalase positive cocci, 19.2% (5/26) coagulase positive and 80.8% (21/26) coagulase negative cocci were detected.

For all Gram-positive cocci isolates loaded into the BD Phoenix™ M50 automatic identification device the following species were detected: 5 (12.2%) *S. pettenkoferi*, 5 (12.2%) *S.*

Table 3. Presentation of PCR study results.

No	BD Phoenix	*nuc*	*coa*	*tuf*	*sea*	*seb*	*sec*	PVL	*Hla*	*Hlb*	*BAP*	TSST	Sequence Analysis
1	*S. pettenkoferi*	–	–	+	+	+	–	+	+	+	–	+	Not Typed
2	*S. epidermidis*	–	–	+	–	–	–	–	+	–	–	–	*S. heamolyticus*
3	*S. aureus*	+	+	–	–	–	–	–	+	+	–	–	*S. aureus*
4	*S. aureus*	+	+	–	–	–	–	–	–	–	–	–	*S. aureus*
5	*S. warneri*	–	–	+	–	+	–	–	+	+	–	+	*S. devriesei*
6	*S. capitis*	–	–	+	–	–	–	–	–	–	+	–	*S. devriesei*
7	*S. equorum*	–	–	+	–	–	–	–	–	–	–	–	*S. succinus*
8	*S. warneri*	–	–	+	–	–	–	–	+	+	–	–	*S. devriesei*
9	*S. equorum*	–	–	+	–	–	–	–	–	–	–	–	*S. succinus*
10	*S. caprae*	–	–	+	–	–	–	–	–	–	–	–	*S.caprae*
11	*S. warneri*	–	–	+	–	–	–	–	–	–	–	–	*S. devriesei*
12	*S. epidermidis*	–	–	+	–	–	–	–	+	–	–	–	Not Typed
13	*S. equorum*	–	–	+	–	–	–	–	–	–	–	–	*S. succinus*
14	*S. caprae*	–	–	+	–	–	+	–	–	–	–	+	*S.caprae*
15	*S. pettenkoferi*	–	–	+	–	–	–	–	–	–	–	–	*S.caprae*
16	*S. pettenkoferi*	–	–	+	–	–	–	–	–	–	–	+	*S. chromogenes*
17	*S. pettenkoferi*	–	–	+	–	–	–	–	–	–	–	–	*S. chromogenes*
18	*S. simulans*	–	–	+	–	–	–	–	+	–	+	–	*S. succinus*
19	*S. epidermidis*	–	–	+	–	+	–	–	+	+	–	+	Not Typed
20	*S. aureus*	+	+	–	–	+	–	–	+	+	–	+	*S. aureus*
21	*S. aureus*	+	+	–	–	–	–	–	+	–	–	–	*S. aureus*
22	*S. pettenkoferi*	–	–	+	–	+	–	–	+	+	–	–	*S. chromogenes*
23	*S. capitis*	–	–	+	–	+	–	–	+	–	–	–	*S. petracii sp. jettensis*
24	*S. caprae*	–	–	+	–	–	–	–	–	–	–	–	*S.caprae*
25	*S. epidermidis*	–	–	+	–	+	+	–	+	+	–	–	Not Typed
26	*S. aureus*	+	+	–	–	+	+	–	+	+	–	–	*S. aureus*

aureus, 4 (9.7%) *S. epidermidis*, 3 (7.3%) *S. equorum*, 3 (7.3%) *S. warneri*, 3 (7.3%) *S. caprae*, 2 (4.9%) *S. capitis*, 1 (2.4%) *S. simulans* and 15 (36.6%) *Streptococcus* sp. were identified. BD Phoenix™ M50 identification results show in Table 3.

PCR

16S rRNA: According to the PCR results, the 16S rRNA gene was detected in all isolates (n=26) (100%) Sequence: *tuf* gene Sanger sequencing results of *Staphylococcus* isolates (n=26) showed that; 5 (19.2%) *S. aureus* [(Access. No: CP087593.2)], 4 (15.4%) *S. caprae* [(Access. No: CP051643.1)], 4 (15.4%) *S. succinus* [(Access. No: MF679024.1; MF679025.1)], 4 (15.4%) *S. devriesei* (Access. No: JX966457.1)], 3 (11.5%) *S. chromogenes* [(Access.No: CP031471.1)], 1 (3.8%) *S. heamolyticus* [(Access.No: CP065356.1)], 1 (3.8%) *S. petracii sp. jettensis* [(Access. No: MF679008.1)]. The four remaining (15.4%) samples could not be typed by the Sanger sequence method. The sequencing results are shown in the Table 3.

For the *Staphylococcus* spp. isolates, the *nuc* gene was detected in 5 (19.2%) isolates, the *coa* gene was detected in 5 (19.2%) isolates, the *tuf* gene was detected in 21 (80.8%) isolates (Table 3.).

The results of the enterotoxin genes PCR procedure on the 26 catalase positive samples showed that the *sea* gene was detected in one (3.8%), the *seb* gene in 8 (30.7%), the *sec* gene in 3 (11.5%) isolates (Figure 1.) (Table 3.).

Virulence genes PCR results of PVL, *Hla* and *Hlb* genes showed that PVL gene was detected in one isolate (3.8%),

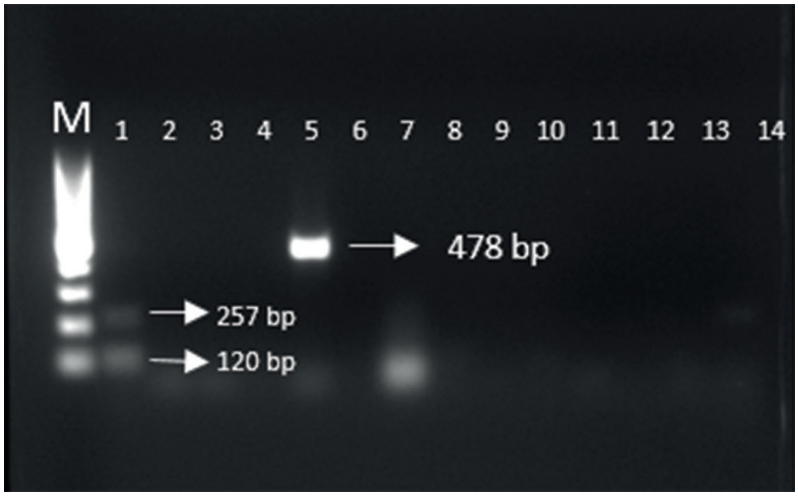


Figure 1. Gel electrophoresis image of *sea*, *seb*, *sec* genes. M: Marker (100 bp DNA Ladder, Fermentas); 1: *sea* (120 bp) and *sec* (257 bp) gene positive sample; 5: *seb* (478 bp) gene positive sample; 2,3,4,6-14; jj*sea*, *seb*, *sec* genes negative samples.

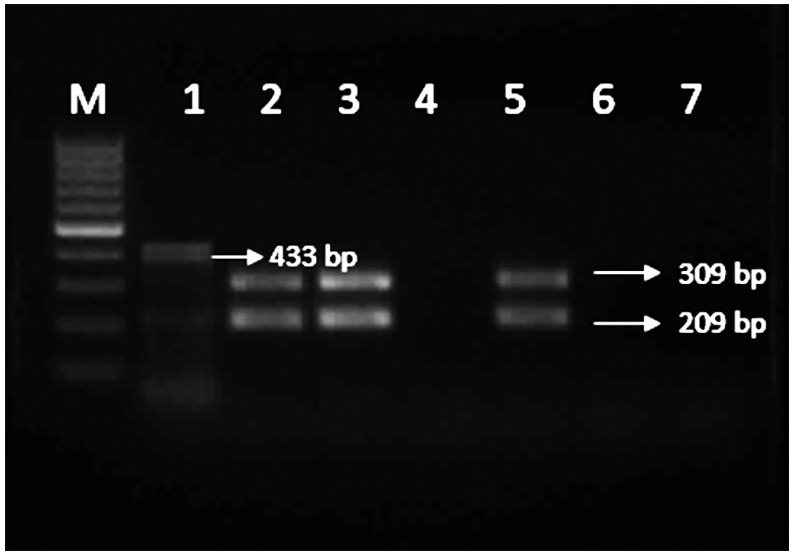


Figure 2. Gel electrophoresis image of PVL, *Hla*, *Hlb* genes. M: Marker (100 bp DNA ladder, Fermentas). 1: PVL (433 bp) and *Hla* (209 bp) gene positive sample; 2, 3 and 5: *Hla* (209 bp) and *Hlb* (309 bp) gene positive samples; 4,6,7: PVL, *Hla* and *Hlb* gene negative samples.

Table 4: The relationship between antibiotic susceptibility and resistance status in isolates carrying and non-carrying coagulase gene.

		Coagulase +	Coagulase –	Total	X^2	P
Penicilin	Resistant	4	19	23	0.43	0.48
	Sensivite	1	2	3		
Ampicillin	Resistant	3	20	23	4.91	0.08
	Sensivite	2	1	3		
Amoxicillin/Clavulanate	Resistant	2	20	22	9.46*	0.01
	Sensivite	3	1	4		

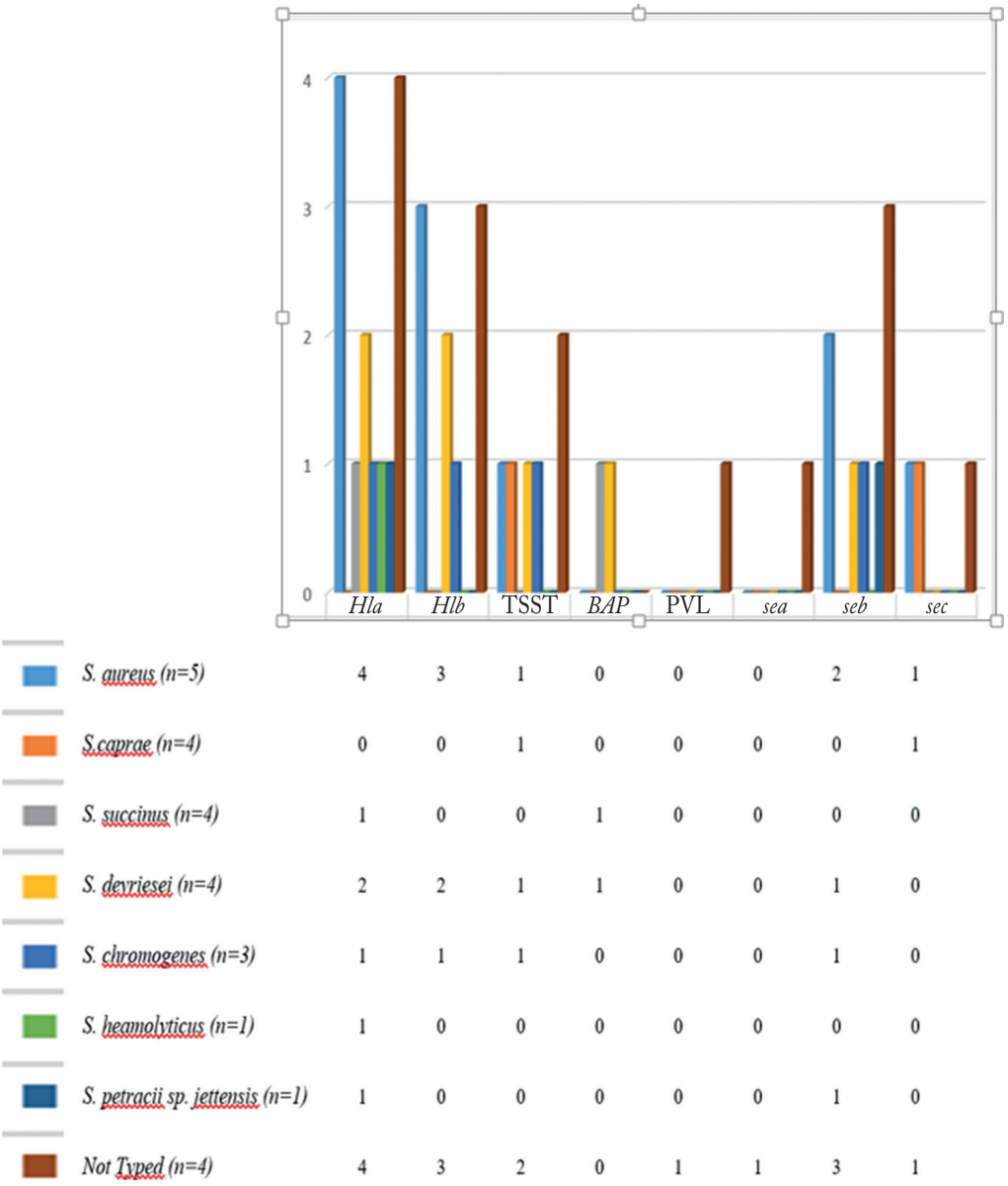


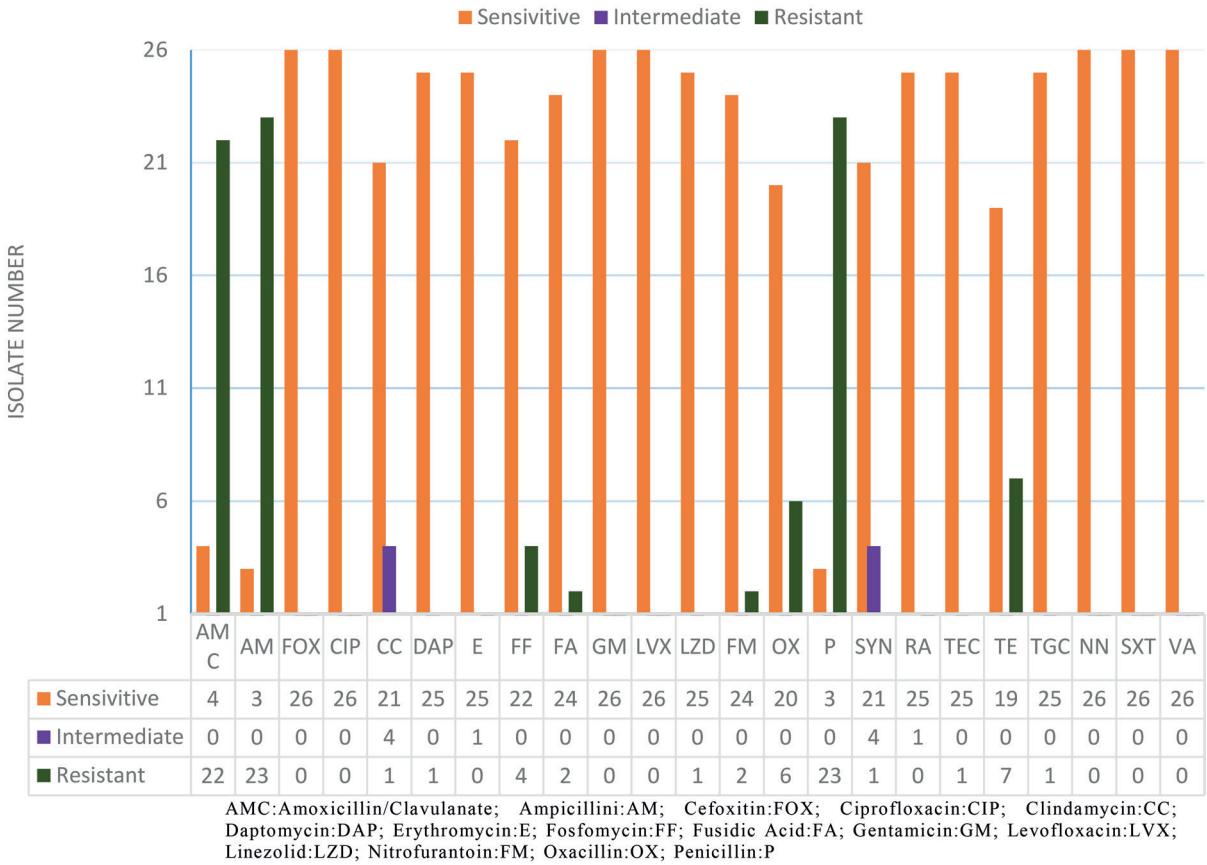
Figure 3. Distribution of virulence and toxin genes in *Staphylococcus* isolates.

Hla gene in 14 (53.8%) and *Hlb* gene in 9 (34.6%) isolates. *BAP* was detected in 2 (7.7%) of 26 isolates. TSST gene was detected in 6 (23.1%) isolates (Figure 2.). PCR results of all genotypically identified samples are shown in (Table 3).

In our research, as a result of the investigation of virulence and toxin genes of *Staphylocccus* isolates by PCR; 80.0% *Hla*, 60% *Hlb*, 20.0% TSST, 40.0% *seb*, 20.0% *sec* genes in *S. aureus* isolates; 25.0% *sec*, 25.0% TSST genes in *S. caprae* isolates; 25.0%*Hla*, 25.0% *BAP* genes in *S. succinus* isolates; 50.0% *Hla*, 50.0% *Hlb*, 25.0% TSST, 25.0% *BAP*, 25.0% *seb* genes in *S. devriesei* isolates; 33.3% *Hla*, 33.3% *Hlb*, 33.3% TSST, 33.3% *seb* genes in *S. chromogenes* isolates; *Hla* and *seb* genes in *S. petracii* sp. *jettensis* isolate were determined. In addition, it was determined that 4 untypable isolates contained 100.0% *Hla*, 75.0% *Hlb*, 75.0% *seb*, 50.0% TSST, 25.0% *BAP*, 25.0% *sec* genes (Figure 3).

Antibiotic susceptibility

According to antibiotic susceptibility results of *Staphylococcus* isolates (n=26) were found to be sensitive at 100% to tobramycin, trimethoprim-sulfomethoxazole, vancomycin, cefoxitin, ciprofloxacin, gentamicin and levofloxacin; to be variably sen-



	AMC	AM	FOX	CIP	CC	DAP	E	FF	FA	GM	LVX	LZD	FM	OX	P	SYN	RA	TEC	TE	TGC	NN	SXT	VA
Sensivitive	4	3	26	26	21	25	25	22	24	26	26	25	24	20	3	21	25	25	19	25	26	26	26
Intermediate	0	0	0	0	4	0	1	0	0	0	0	0	0	0	0	4	1	0	0	0	0	0	0
Resistant	22	23	0	0	1	1	0	4	2	0	0	1	2	6	23	1	0	1	7	1	0	0	0

AMC:Amoxicillin/Clavulanate; Ampicillini:AM; Cefoxitin:FOX; Ciprofloxacin:CIP; Clindamycin:CC; Daptomycin:DAP; Erythromycin:E; Fosfomycin:FF; Fusidic Acid:FA; Gentamicin:GM; Levofloxacin:LVX; Linezolid:LZD; Nitrofurantoin:FM; Oxacillin:OX; Penicillin:P

Figure 4. Antibiotic susceptibility result graph of *Staphylococcus* isolates (n=26)

sitive at 73-96% to clindamycin, daptomycin, erythromycin, fosfomycin, fusidic acid, linezolid, nitrofurantoin, oxacillin, quinupristin-dalfopristin, rifampin, teicoplanin, tetracycline, tigecycline; and to be sensitive at the range of 11.5-15.3% to ampicillin, penicillin and amoxicillin-clavulanic acid. The antibiogram results are shown in Figure 4.

Statistical analyses

The relationship between antibiotic resistance and coagulase production, are presented in Table 4. There was no significant relationship between the some antibiotics (penicillin and ampicillin) resistance of the isolates and the coagulase production. However, the relationship between the amoxicillin/clavulanate resistance and the coagulase production was significant ($p<0.01$).

DISCUSSION

Mastitis in dairy animals is a disease that should be considered seriously due to its economic implications, as it negatively affects milk quality, milk yield and animal welfare. As in cows, udder health, milk yield and quality of sheep and goats are of great importance for the profitability of the producers. Mastitis is the most important factor affecting udder health, milk yield and quality. Bacteria can cause chronic and subclinical intramammary infections, albeit at small rates, and in this respect, they are important for udder health in small ruminant enterprises. Mastitis is especially important in two respects: Bacteria in milk that has not been sufficiently heat treated or antibiotics used in the treatment of mastitis can be transmitted to humans through the food chain and threaten public health. The bacterial species that cause mastitis in goats are mainly *Staphylococcus* genus (20). CNS, previously considered as saprophytes and non-pathogenic contaminating species in clinical stages, have been included in the literature as a very important source of infection in the last 30 years. In caprine the CNS are the most prevalent microorganisms (ranging from 25 to 93), and are isolated mainly from subclinical infections (21, 22).

Jabbar *et al.* (2020) reported that the overall prevalence of mastitis was 309 (61.8%) in 500 in Beetal goats, and 260 (52%)

of them were positive for subclinical mastitis by CMT test. Gram (+) cocci isolate ratios for SCM positive samples were *Staphylococcus* 15.0%, *Streptococcus* 86.5% percentages, respectively (23). In our research, Gram positive *Staphylococcus* isolated were 23.6% (26 to 110 samples) from goat mastitis milk.

Mahlangu *et al.* (2018) found that the prevalence of subclinical mastitis in 110 goat milk was determined to be 50.9% using CMT, and 86.5% of these yielded bacteria in culture (24). Compared with our study, Mahlangu *et al.* (2018) for Gram positive cocci; CNS (20.7%), *S. aureus* (10.7%), *Streptococcus* spp. (1.2%), *Staphylococcus intermedius* (0.6%) were isolated and identified from the samples. In our study, we isolated 12.2% *S. aureus*, 51.2% CNS and 36.6% *Streptococcus* sp. from subclinical mastitis. We also isolated more CNS bacteria in subclinical mastitis cases.

In the study conducted by Moroni *et al.* (2005), a total of 1,367 aseptic foremilk samples were collected from both half udders of 88 lactating Alpine goats. The most common mastitis agents were CNS (n=320 samples) represting 23.4%. *Staphylococcus aureus* was isolated in 20.8% (n=284) as the second most common pathogen (25). We isolated 4.5% *S. aureus* from all goat mastitis samples.

Rocío *et al.* (2018) stated that 53 *Staphylococcus* strains were isolated from the milk of both healthy goats and goats with subclinical mastitis. Isolates were Gram stained and catalase tested to identify *Staphylococcus* sp. They used the API Staph® (V4.1) system to identify coagulase negative strains. From the results of the API system Staph®, 23 isolates corresponded to *S. chromogenes*, 18 isolates *Staphylococcus simulans*, 9 isolates *Staphylococcus xylosus*, 2 isolates *S. sciuri* and 1 isolate *Staphylococcus warneri* (26).

Salaberry *et al.* in their research (2015), out of 226 caprine subclinical mastitis milk samples 124 (54.9%) were positive sample *Staphylococcus* spp., 101 isolates (81.5%) were CNS and 23 (18.5%) isolates were CPS. The CNS species most frequently isolated were *S. epidermidis* (22.6%), *S. lugdunensis* (13.7%), *S. chromogenes* (9.7%), *S. capitis* (8.1%), *S. hominis* (6.5%) and *S. auriculares* (5.7%) and *S. xylosus* (5.7%). CPS isolates identified; *S. intermedius* (12.1%) and *S. aureus* (6.5%) (27).

In our study, forty-one (37.2%) Gram-positive cocci were isolated from the 110 goat milk samples and found to be CNS (51.2%) which were primer bacterial agent in Saanen goat mastitis. The use of molecular-based techniques for identification was used to clarify the specificity and sensitivity offering information regarding the identification and genotyping of *Staphylococcus* species. Heikens *et al.* (28) pointed out the importance of 16S rRNA and *tuf* gene in their study. Bes *et al.* (29) stated that the most suitable region for DNA sequencing among bacterial genes is the 16S rRNA gene, Lan and Reeves (30) used the 16S rRNA gene in the genotypic identification of 263 CNS isolates in their study for the identification and serovar typing of CNS derived from ruminants. Vanderhaeghen *et al.* (31) used the *tuf* primary sequence for genotypic identification of CNS from 98 milk samples with subclinical mastitis. Heikens *et al.* (28) reported that *tuf* gene identification was more stable in CNS identification compared to 16S rRNA by using the *tuf* gene, which contains the Tu factor that determines expansion during amplification, to differentiate CNS from *Staphylococcus*. Ghebremedhin *et al.* (32) found 96-99% positivity for 16S rRNA in different *Staphylococcus* species. In our study, all of *Staphylococcus* isolates were confirmed 16S rRNA PCR. Then *nuc*, *coa*, *tuf* gene specified PCR examined 26 *Staphylococcus* isolates and identified 5 isolates as *S. aureus* of which 21 isolates were CNS.

In our research, *Staphylococcus* isolates genotypic identifications (*tuf* gene Sanger sequencing typing) were made together with the phenotypic identifications, aimed to confirm the *Staphylococcus* isolates. The *tuf* gene Sanger sequencing identified 5 (12.2%) *S. aureus*, 5 (12.2%) *S. pettenkoferi*, 4 (9.7%) *S. epidermidis*, 3 (7.3%) *S. equorum*, 3 (7.3%) *S. warneri*, 3 (7.3%) *S. caprae*, 2 (4.9%) *S. capitis*, 1 (2.4%) *S. simulans* and 15 (36.6%) *Streptococcus* spp. BD Phoenix™ 50 device identified 5 (19.2%) *S. aureus*, 4 (15.4%) *S. caprae*, 4 (15.4%) *S. succinus*, 4 (15.4%) *S. devriesei*, 3 (11.5%) *S. chromogenes*, 1 (3.8%) *S. heamolyticus*, 1 (3.8%) *S. petracii sp. jettensis*.

With these results, it appeared that the phenotypic typing of 19 (73.0%) isolates and the sequence typing were dissimilar. When we compared the phenotypic and genotypic results, only the identification results of *S. aureus* and *S. caprae* isolates were compatible with each other. The similarity of the results of the biochemical tests used in phenotypic isolation methods may cause different identifications. However, the sequence method is a DNA-based method and gives precise results. Therefore, differences may occur between phenotypic typing and sequence typing.

Alpha hemolytic toxin often acts by attaching to transmembrane pores and breaking down erythrocytes. As with β-hemolysis, phospholipase activity specific to sphingomyelin

acts by causing partial cell lysis (33). Specific for *Hla*, *Hlb* genes in this study. As a result of PCR performed, 14 (53.8%) *Staphylococcus* isolates detected α-hemolysis positive and 9 (34.6%) β-hemolysis positive.

Rana *et al.* (2020) in goat milk with mastitis research, 49 (73.1%) samples were found positive for *Staphylococcus*, they determined that 17 (34.7%) isolates carried the *nuc* gene by PCR analysis, thus they were defined as Coagulase positive staphylococci and 32 (65.3%) isolates were found to be CNS (22). Salaberry *et al.* (2015) reported that 124 *Staphylococcus* sp. as a result of the *BAP* gene PCR analysis performed on the isolates, 58 (46.8%) isolates were positive (27). In our study, the *Hla*, *hlb*, *seb*, TSST, *sec*, *BAP*, PVL, *sea* virulence and toxin genes were determined in 14, 9, 8, 6, 3, 2, 1, 1 *Staphylococcus* isolates, respectively.

Salaberry *et al.* (2015), reported that, concerning the enterotoxins genes; 12 (9.7%) isolates were positive for *sea*, 13 (10.5%) for *seb*, 17 (13.7%) for *sec*, 30 (24.2%) for *see* and 35 (28.2%) for *sed* (27). The genes *sed* and *see* were the most frequently detected. Compared to our study results the most frequently detected enterotoxin gene was *seb*. The gene *seb* was more frequently detected in CPS when compared with CNS. In our study, the presence of the *sea*, *seb*, *sec* genes was detected by multiplex PCR performed with primers specific for the *sea*, *seb*, *sec* genes.

Although antibiotics are used in the treatment and prophylaxis of mastitis caused by bacteria, they may not show the expected effect in treatment and prevention because of the antimicrobial resistance. In a previous study, staphylococcal species isolated were reported resistant to 72.7% to amoxicillin, 34.9% to ampicillin, 1.5% to cefoxitin, 1.5% to gentamicin, 16.7% to oxacillin, 40.9% to penicillin, 12.1% to tetracycline, 12.1% to vancomycin (34).

Penicillinase enzymes, also called lactamases, act by breaking down some lactam antibiotics along with penicillin. Santos *et al.* (34) found for different beta-lactams in *Staphylococcus* isolates of goat mastitis: the MIC_{50} values ranged from a minimum of 4 μg/mL to a maximum of 64 μg/mL whereas MIC_{90} values ranged from a minimum 8 μg/mL to a maximum 128 μg/mL.

Virdis *et al.* (2010) reported that, *S. aureus* susceptibility was lower for kanamycin, oxytetracycline, and ampicillin from the ten antibiotics tested. CNS sensitivity was slightly lower for ampicillin and kanamycin. *Staphylococcus* spp. isolates showed poor susceptibility to AMP. Among the AMP-resistant CNS, the most common strains were *S. caprae* (37.0%) and *S. chromogenes* (22.2%), while only 12.0% of *S. aureus* strains were resistant. Cephalosporins showed high activity against *Staphylococcus* spp. isolates.

The aim of this study was to identify *Staphylococcus* species by conventional and molecular methods from Saanen goat milk with mastitis and to determine the virulence genes and the antibiotic susceptibility of the isolates. *Staphylococcus* spp. were isolated at a rate of 23.6% in our study. Both phenotypic and genotypic identifications of these staphylococcal isolates were examined. Because of the *tuf* gene sequence typing, *S. aureus*, *S. caprae*, *S. succinus*, *S. devriesei*, *S. chromogenes*, *S. heamolyticus*, *S. petracii* sp. *jettensis* was identified and 4 *Staphylococcus* isolates could not be typed. As a result of the antibiogram tests performed on staphylococcal isolates, it is found that resistance development has occurred to penicillin, amoxicillin and amoxicillin+clavulanic acid. Sensitivity to cefoxitin, ciprofloxacin, gentamicin, levofloxacin, tobramycin, trimethoprim/sulfamethoxazole, vancomycin antibiotics were detected.

Based on the results of this study, we consider that analysis of the *tuf* gene is an accurate tool for the identification of CNS strains. Finally, the use of advanced diagnostic methods in goat mastitis cases and the determination of antibiotic susceptibility of *Staphylococcus* isolates will prevent the occurrence of results that may harm public health.

ACKNOWLEDGEMENTS

This manuscript was supported by Aydin Adnan Menderes University Scientific Research Projects Unit (Project Number: VTF-19034).

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