Molecular Characterization of *Staphylococcus aureus* from Clinical Sheep Mastitis Cases

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ABSTRACT

In the current study, it was aimed to examine the genes encoding toxin, biofilm and microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) of 62 *Staphylococcus aureus* isolated from sheep clinical mastitis cases using polymerase chain reaction (PCR). Besides, accessory gene regulator (*agr*) groups and the antibiotic susceptibilities of the isolates were also determined. Most of the isolates (87.1%) were susceptible to all tested antimicrobials. The *sec* (38, 61.3%) and *sell* (50, 80.6%) were the only staphylococcal enterotoxin (SE) genes detected among the isolates. The *tst* gene was observed in 43 (57.3%) isolates. Whereas co-existence of *sec*, *sell*, *tst* was detected in 36 (48%) isolates; none of them isolates harbored *eta* and *etb* genes. However, eight (12.9%) isolates were found to be negative for SE, ET and TSST-1 genes. Hemolysin genes were detected in all isolates and the frequency of *hla*, *hlb*, *hld*, and *hlg*2 were 100%, 95.2%, 98.4%, and 61.3%, respectively. The *luk*ED and *luk*M were found together in 40 (64.5%) of the isolates, and *luk*ED was found alone in 22 (35.5%) of the isolates. Of the biofilm-related genes, i*ca*D was the only gene detected with a frequency of 83.9% (52). MSCRAMMs genes *eno*, *cna*, *ebps, fib*, *fnb*, *clf*A, and *clf*B were detected in 100%, 79%, 100%, 95.2%, 100%, and 100%, respectively. None of the isolates carried the *bbp* gene. This study indicated that *S. aureus* isolates from clinical sheep mastitis possessed several virulence-associated determinants.

Keywords: Biofilm; Clinical Mastitis; Sheep; *Staphylococcus aureus*; Toxin.

INTRODUCTION

In the dairy industry, mastitis is considered one of the most common and economically important diseases worldwide, due to reduced milk quality and production, milk discard, involuntary culling, veterinary services, treatment costs and increased labor expenses (1). Mastitis is a multifactorial disease, involving interrelationship between microorganisms, host and environmental factors, and it is, therefore, necessary to elucidate bacteria-host interactions (2). Although a wide range of microorganisms may cause sheep mastitis, staphylococci are the main etiological agents isolated from mastitic milk samples, and *Staphylococcus aureus* is a frequent cause of clinical mastitis cases (3).

As a pathogen, the success of *S. aureus* infections is related to the expression of several virulence factors. These include staphylococcal enterotoxins (SEs), toxic syndrome toxin 1 (TSST-1), exfoliative toxins (ETA and ETB), hemolysins, and leukocidins (4). According to the emetic activity in humans or non-human primates, SEs have been divided into two main groups; staphylococcal enterotoxins (SEs) and newly identified staphylococcus-like proteins (SEls). To date, there are more than 23 different SEs/SEls with amino acid sequence features, ranging between 21% and 83% (5). SEs together with TSST-1 are known staphylococcal superantigens (SAg) due to their superantigenic activity. These SAg toxins have been reported to cause aggravation of clinical conditions in sheep mastitis (3). The fact that SEs/SEls are carried on

various mobile genetic elements (MGE), such as prophages, transposons, plasmids, and *S. aureus* pathogenicity islands (SaPIs), leading to the widespread transfer of these genes among staphylococci. In addition, SEs, when consumed with high doses by susceptible individuals, cause staphylococcal food poisoning (SFP) characterized by symptoms such as vomiting, abdominal cramps, and sometimes diarrhea (5).

S. aureus produces a variety of exotoxins with cytolytic activities such as α-, β-, δ-, and γ-hemolysins, leukocidins (*lukED* and *luk*M) and Panton-Valentine leukocidin (PVL) that cause the destruction of erythrocytes and leukocytes by the formation of β-barrel-channel pores on the target cell membranes (6).

Expression of most virulence factors including several cell-wall-associated and secreted extracellular proteins in *S. aureus* are controlled via the accessory gene regulator (*agr*) locus (7). The *agr* system shows a dual action on the regulation of virulence according to the presence or absence of environmental signals (e.g., autoinducing peptide, reactive oxygen species (ROS), high cell density, nutrient availability, and glucose concentration). Its effects include up-regulation of genes associated with invasive infection (e.g., TSST-1, enterotoxins, serin proteinase, lipases) and simultaneous down-regulation of genes associated with colonization (e.g., adhesins) (3, 8). To date, there are four described allelic variants (*agr*A/B/C/D) of the *agr* system (9).

The increasing trend of antimicrobial resistance presents a growing burden for the prevention and treatment of mastitis due to widespread and misuse of antimicrobials. The emergence of antimicrobial resistance among mastitis pathogens is also a concern for public health because resistant bacteria can also be transmitted to humans through the food chain (10).

The biofilm forming ability of *Staphylococcus* spp. has been increasingly considered as an important virulence factor that facilitates adhesion and colonization on the mammary gland epithelium (11, 12). Biofilm comprises bacterial cells enclosed in a self-produced matrix attached to biotic or abiotic surfaces (13). Biofilm gives many advantages to the bacteria such as (i) protection from the immune system and hostile environments within the host; (ii) decreasing diffusion of bactericidal concentrations of antibiotics or disinfectants inside biofilm matrix; (iii) helping bacteria for adhesion and colonization on mammary gland tissue and persistence of infection (14).

Staphylococcal biofilm formation is a two-step process involving cell attachment and the formation of an extracellular matrix. Staphylococci can express a variety of bacterial surface molecules called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) interacting with host tissues like fibronectin-binding proteins A (*fnb*A) and B (*fnb*B), clumping factors (*clf*A) A and B (*clf*B), bone sialoprotein-binding protein (*bbp*), elastin-binding protein (*ebpS*), laminin-binding protein (*eno*), collagen-binding protein (*cna*), and fibrinogen-binding protein (*fib*) (15, 16). The next step is the development of biofilm, facilitated by the polysaccharide intercellular adhesin (PIA), called poly-N-acetylglucosamine (PNAG). PIA synthesis results in multilayer cell clustering. PIA synthesis is regulated by the *ica* gene locus, consisting an N-acetylglucosamine transferase (*ica*A and *ica*D), a PIA deacetylase (*ica*B), a putative PIA exporter (*ica*C), and a regulatory gene (*ica*R) (17). However, it has been reported that PIA production is not mandatory for biofilm formation and biofilm-related infection (18), and when there are no *ica* genes in some strains isolated from biofilm-related infections (19).

In Turkey, research related to clinical sheep mastitis is very limited compared to the studies on bovine mastitis. This study was therefore conducted (i) to determine the antimicrobial susceptibilities and *agr* types of the isolates, (ii) to examine genes encoding SE, TSST-1, ET, leukocidin and hemolysin, (iii) to evaluate biofilm-forming ability, and (iv) to investigate the genes encoding biofilm and MSCRAMMs.

MATERIALS AND METHODS

Study area

Şanlıurfa is located in southeastern Turkey, with an altitude of 508.08 m (37° 10' 1.49" N – 38° 47' 38.11" E). It has a border with Syria in the south (Figure 1). Summers are hot and arid, and winters are colder than those of the Mediterranean climate. Şanlıurfa receives about 64.34 mm rain precipitation, and annual rainy days are 88.73 mm.

Sampling

A total of 240 milk samples were collected from Awassi ewe herds, at the age of 2-5 years, with clinical mastitis problems in eight villages of Şanlıurfa of Turkey between March 2019 and August 2020, which did not receive antibiotic treatment.

Sampling was caried out by cleaning the teats by using 70% alcohol. Subsequently, the first few streams of foremilk

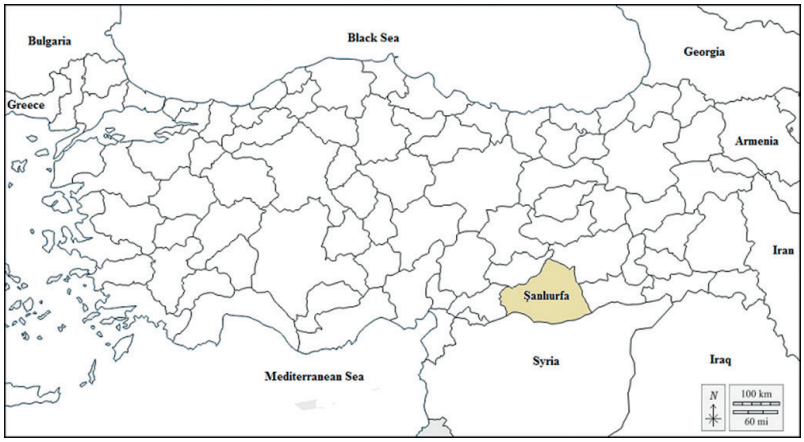

Figure 1. Map depicting the province that *S. aureus* isolates were obtained

were discarded, the milk samples were aseptically collected into sterile tubes.

S. aureus isolates

Each of the milk samples were inoculated onto Blood Agar (Merck, Darmsadt, Germany) supplemented with 5% defibrinated sheep blood, and then aerobically incubated at 37 °C for 18-24 hours. Following conventional microbiological methods such as colony morphology, Gram staining, hemolysis, catalase, and coagulase (20); species identification was carried out using MALDI-TOF MS (Bruker Daltonics, Billerica, MA, United States) and *nuc* gene based PCR (21). The isolates were stored at −80°C by using the cryobank system (MAST Group Ltd., Bootle, United Kingdom).

DNA isolation

Genomic DNA isolation from *S. aureus* isolates was performed using a commercial extraction kit DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). Before extraction, for lysis of the cell wall of the isolates, 10 µl of lysostaphin (10 mg/ml) and 15 µl of lysozyme (10 mg/ml) were added to the bacterial suspension and incubated at 37°C for 45 minutes (22).

Antimicrobial susceptibility testing

Antimicrobial susceptibilities of the isolates were performed and evaluated according to Clinical Laboratory Institute (CLSI) criteria using disc diffusion method (23). The following antimicrobial discs were used: penicillin (P, 10 U), oxacillin (OXA, 1 µg) ampicillin (AMP, 10 µg), amoxicillin-clavulanic acid (AMC, 20/10 µg), cefoxitin (FOX, 30 µg), gentamicin (CN, 10 µg), tetracycline (TE, 30 µg), ciprofloxacin (CIP, 5 µg), chloramphenicol (C, 30 µg), and erythromycin (E, 15 µg). Antimicrobial discs were purchased from Bioanalyse (Ankara, Turkey).

S. aureus ATCC 25213 was used as a control strain. This strain was obtained from Public Health Institution of Turkey.

Detection of virulence genes

PCR detection of SE (*sea, seb, sec, sed, see, seg, seh, sei, selj, selk, sell, seln, selm, selo, selq, selr*), TSST-1 (*tst*), ET (*eta*A and *eta*B), PVL (*pvl*), leukocidin (*luk*E-*luk*D, *luk*M), and hemolysin (*hla*, *hlb*, *hld*, *hlg*) genes were carried out as previously described (7, 24, 25, 26).

Screening of MSCRAMMs and Biofilm-related genes

Biofilm-related (*ica*A, *ica*D, and *bap*) and MSCRAMM genes (*ebpS*, *eno*, *cna*, *fnbA*, *fnbB*, *fib*, *clfA*, *clfB*, and *bbp*) were searched as previously reported (3, 27, 28).

agr Typing

The *agr* groups of the isolates were determined by mPCR as previously reported by Gilot *et al.* (9).

Determination of biofilm formation

Two different methods investigated the determination of biofilm formation: Phenotypic biofilm-forming ability of the: (i) standard tube (ST) method and (ii) microtiter plate (MTP) method.

Standard Tube (ST) Method: The qualitative characterization of biofilm formation was performed as previously described by Christensen *et al.* (29). The presence of adher-

ent film stained with safranine on the inner surface of the standard tubes (ST) was considered as an indication of a positive result. The biofilm formation was scored as negative (-), weak (+), moderate (++), or strong (+++).

Microtiter Plate (MTP) Method: Quantitative biofilm determination was carried out using the microtiter plate (MTP) method as previously described by Stepanovic *et al*. (30) in tissue culture plates with 96 flat-bottomed wells. Every isolate was tested in triplicate, and un-inoculated Tryptone Soya Broth (Merck, Darmstadt, Germany) was used as a negative control. The amount of the biofilm formation in each well was measured using a microplate ELISA reader (BioTek, Quant, USA) at 570 nm. Based on the optical densities of each well, the isolates were defined as non-producer, weak, moderate or strong.

RESULTS

A total of 62 *S. aureus* were isolated from the examined milk samples. Most of the isolates were susceptible to nearly all tested antimicrobials. Only a small number of the isolates revealed resistance to P, AMP, AMC, TE, and CN with a frequency of 11.3%, 11.3%, 4.8%, 3.2% and 1.6%, respectively.

Of the 17 investigated SE/SEl genes, *sec* were detected in 61.3% (38/62) and *sell* in 80.6% (50/62) of the isolates. Six (14.5%) isolates were negative for SE/SEI genes. The *tst* gene was detected in 43 (69.4%) isolates. None of the isolates carried ET genes.

All the isolates carried the *luk*ED and *luk*M genes as singles or in combination. While these two genes were detected in 44 (71%) isolates together, 18 (29%) isolates were positive for *luk*ED gene alone. None of the isolates harbored *pvl* gene.

The hemolysin genes were detected in varying combinations in all isolates. The frequency of hemolysin genes of *hla*, *hlb*, *hld* and *hlg*2 were 100%, 95.2%, 98.4% and 61.3%, respectively.

While all 62 *S. aureus* isolates were found as biofilm producers by ST method, 61 isolates were positive for biofilm-forming ability by MP method. Comparison of ST and MP methods are given in Table 1.

Concerning the biofilm related genes, while *ica*A and *bap* genes were absent in all isolates, 83.9% (52/62) of the isolates were found to possess for i*ca*D. Of the MSCRAMM genes investigated, *eno*, *cna*, *ebps, fib*, *fnb*, *clf*A, *clf*B were detected in 62 (100%), 49 (79%), 62 (100%), 59 (95.2%), 6 (9.7%), 62 (100%), and 62 (100%) isolates, respectively. None of the isolates carried *bbp* gene. Compiled results of antimicrobial susceptibility, virulence, biofilm and MSCRAMMs among the isolates are presented in Table 2.

Table 1. *Screening of 62 S. aureus isolates for biofilm production by standard tube (ST) and microplate (MP) methods.*

Biofilm production	ST n(%)	MTP n(%)
High	11 (17.74)	10 (16.3)
Moderate	50 (80.64)	52 (83.87)
Weak/non	1 (1.61)	0 (%)
Total	**62 (100)**	**62 (100)**

DISCUSSION

In the current study, *S. aureus* isolates from clinical sheep mastitis cases were subjected to analysis for several potential virulence factors involved in the pathogenesis of udder infection of sheep.

The antimicrobial susceptibility testing revealed that 87.1% (8/62) of the isolates were susceptible to all antibiotics tested. Previously, a low frequency of antimicrobial resistance has been reported in Italy and Algeria (31, 32).

S. aureus is a versatile pathogen that causes a wide range of infections in both humans and animals. This agent has the ability to produce several virulence factors such as exotoxins, exoenzymes, and adhesins (3, 6, 7). Of the SAg genes examined, the *sec* gene was detected in 38 (61.3%) *S. aureus* isolates. Although previous studies have shown the rare occurrence of other SE genes, *sec* was reported to be the most common SE gene detected in *S. aureus* isolates from sheep mastitis (31, 32). Similar to these studies, *sec* was detected in 34 isolates together with *sell* and *tst*. Merz *et al.* (33) reported that the prevalence of *sec*, *sell*, and *tst* genes were significantly higher among small ruminant isolates than the bovine isolates. The co-existence of these genes indicated the presence of two *S. aureus* pathogenicity islands such as SaPIbov1 and SaPIn1/m1, which carry genes encoding SEC, SEIL and TSST-1 (34). Horizontal transfer of MGEs carrying SAg genes has been found to lead to the emergence of new *S. aureus* lineages (35). In addition, single or various combinations of these genes were also observed among SAg positive isolates such as *sec-sell* (in one isolate), *tst-sell* (in 7 isolates), *sec-tst* (in one isolate), *sec* (in one isolate) and *sell* (in 7 isolates). The occurrence of the isolates harboring *sec*, *tst* and *sell* genes

Table 2. Results of antimicrobial susceptibility and virulence genes encoding toxin, biofilm and MSCRAMMs.

Number of isolates	Resistance profiles	Toxin gene profile	Hemolysins	Leukocidins	*ica*D	MSCRAMM genes
1	P, AMP, AMC	*tst*, *sell*	*hla*, *hlb*, *hld*, *hlg-2*	*luk*ED, *luk*M	*ica*D	*cna*, *eno*, *ebp*S, *fib*, *clf*A, *clf*B
1	P, AMP, AMC	(-)	*hla*, *hlb*, *hld*, *hlg-2*	*luk*ED	(-)	*cna*, *eno*, *ebp*S, *fnb*B, *fib*, *clf*A, *clf*B
1	P, AMP, AMC	(-)	*hla*, *hlb*, *hld*	*luk*ED	(-)	*eno*, *ebp*S, *fnb*B, *fib*, *clf*A, *clf*B
1	P, AMP, TE	*sec*	*hla*, *hlb*, *hld*, *hlg-2*	*luk*M, *luk*ED	*ica*D	*cna*, *eno*, *ebp*S, *fib*, *clf*A, *clf*B
1	P, AMP, TE	(-)	*hla*, *hlb*, *hld*	*luk*ED, *luk*M	(-)	*eno*, *ebp*S, *fib*, *clf*A, *clf*B
1	P, AMP	(-)	*hla*, *hlb*, *hld*	*luk*ED	*ica*D	*eno*, *ebp*S, *fib*, *clf*A, *clf*B
1	P, AMP	(-)	*hla*, *hlb*, *hld*	*luk*ED	(-)	*eno*, *ebp*S, *fib*, *clf*A, *clf*B
1	CN	*sec, tst*, *sell*	*hla*, *hlb*, *hld*, *hlg-2*	*luk*ED, *luk*M	*ica*D	*cna*, *eno*, *ebp*S, *fib*, *clf*A, *clf*B
22		*sec, tst*, *sell*	*hla*, *hlb*, *hld*, *hlg-2*	*luk*M, *luk*ED	*ica*D	*cna*, *eno*, *ebp*S, *fib*, *clf*A, *clf*B
3		*sec, tst*, *sell*	*hla*, *hlb*, *hld*	*luk*ED, *luk*M	*ica*D	*cna*, *eno*, *ebp*S, *fib*, *clf*A, *clf*B
2		*sec, tst*, *sell*	*hla*, *hlb*, *hld*	*luk*ED	*ica*D	*cna*, *eno*, *ebp*S, *fib*, *clf*A, *clf*B
3		*sec, tst*, *sell*	*hla*, *hlb*, *hld*, *hlg-2*	*luk*ED	*ica*D	*cna*, *eno*, *ebp*S, *fib*, *clf*A, *clf*B
3		*sec, tst*, *sell*	*hla*, *hlb*, *hld*, *hlg-2*	*luk*ED, *luk*M	(-)	*cna*, *eno*, *ebp*S, *fib*, *clf*A, *clf*B
1		*sec, tst*	*hla*, *hlb*, *hld*, *hlg-2*	*luk*ED, *luk*M	*ica*D	*cna*, *eno*, *ebp*S, *fib*, *clf*A, *clf*B
1		*sec*, *sell*	*hla*, *hlb*, *hld*	*luk*ED	(-)	*cna*, *eno*, *ebp*S, *fib*, *clf*A, *clf*B
3		*tst*, *sell*	*hla*, *hlb*, *hld*, *hlg-2*	*luk*ED, *luk*M	*ica*D	*cna*, *eno*, *ebp*S, *fib*, *clf*A, *clf*B
4		*tst*, *sell*	*hla*, *hlb*, *hld*	*luk*ED	*ica*D	*cna*, *eno*, *ebp*S, *fib*, *clf*A, *clf*B
1		*sec*	*hla*, *hlb*, *hld*, *hlg-2*	*luk*ED	*ica*D	*eno*, *ebp*S, *fnb*B, *fib*, *clf*A, *clf*B
2		*sell*	*hla, hld*	*luk*M, *luk*ED	*ica*D	*eno*, *ebp*S, *clf*A
3		*sell*	*hla*, *hlb*, *hld*	*luk*ED	*ica*D	*cna*, *eno*, *ebp*S, *fib*, *clfA*, *clf*B
1		*sell*	*hla*, *hlb*, *hld*, *hlg-2*	*luk*ED	*ica*D	*cna*, *eno*, *ebp*S, *fnb*B, *fib*, *clf*A, *clf*B
1		*sell*	*hla*, *hlb*, *hld*	*luk*ED	(-)	*eno*, *ebp*S, *fnb*B, *fib*, *clf*A, *clf*B
2		(-)	*hla, hlb, hld, hlg-2*	*luk*ED, *luk*M	*icaD*	*cna, eno, ebpS, fib, clfA, clfB*
1		(-)	*hla, hld, hlg-2*	*luk*ED	*ica*D	*eno*, *ebp*S, *fib*, *clf*A, *clf*B
1		(-)	*hla*, *hlb*, *hld*	*luk*ED	(-)	*eno*, *ebp*S, *fnb*B, *fib*, *clf*A, *clf*B

alone or in combination suggest the possibility of the existence of variations or new types of mobile genetic elements (36). Although the role of SAgs in intramammary infections remains to be fully elucidated, they allow *S. aureus* to colonize in mammary glands, and subsequently to cause tissue damage through modulation of the immune response (13).

The development of biofilm by staphylococci is related to the synthesis of PIA, controlled by the *ica*ADBC operon (17). However, staphylococci have been reported to be able to form biofilm in the absence of PIA, and cause biofilm-related infections. This indicates the existence of certain surface proteins contributing to bacterial adhesion and biofilm formation (37, 38). In *S. aureus* strains isolated from mastitis cases, the frequency of genes related to adhesion and biofilm synthesis has been reported to vary according to the studied geogrophical areas (31, 32). In this study, even nearly all *S. aureus* isolates showed biofilm-forming ability, only the *ica*D gene (83.9%, 52/82) was detected in *S. aureus* isolates, but none of the isolates was positive for *ica*A and *bap* genes. Azara *et al*. (32) investigated biofilm-forming ability of 258 *S. aureus* from sheep mastitis by Congo Red Agar (CRA) method as well as for the presence of *ica*A and *ica*D genes and found all isolates being negative. On the other hand, Achek *et al*. (31) detected *ica* (A, C, D) genes in all of the isolates, except *bap* gene. Darwish and Asfour (39) and Tremblay *et al*. (40) also indicated that the process of biofilm formation is a complex phenomenon involving many genes or many unknown factors.

S. aureus has the ability to express cell surface protein receptors designated MSCRAMMs, which are implicated in adhesion and colonization on host cells that is a critical step at the onset of the infection (41). In the present study, all isolates carried MSCRAMMs genes with 6 different profiles, being the most common were *cna*, *eno*, *ebp*S, *fib*, *clf*A, and *clf*B (in 47 isolates). The presence of various of MSCRAMMs genes observed in *S. aureus* strains can be accepted as evidence for providing a selective advantage for better host colonization.

Among the toxins produced by different *S. aureus* strains, bicomponent leukotoxins are pore-forming toxins, targeting PMNL and macrophages, that interfere with cellular immunity of the mammary gland (42). Cytotoxic and antiphagocytic properties of the invading bacteria result in exacerbation of severity of infection in the mammary gland (43). In the present study, PCR analysis of the isolates for leukotoxins revealed the simultaneous presence of *luk*ED and *luk*M in 44 (71%) isolates and only *luk*M gene in 18 (29%) isolates was detected. However, none of the isolates harbored *pvl* gene. Azara *et al*. (32) reported a prevalence rate of 93.4% (241/258) among *S. aureus* isolates with various combinations, *luk*M-*luk*ED-*luk*PV83 (159/258, 61.6%), followed by *luk*M-*luk*PV83 (33/258, 12.8%), *luk*M (20/258, 7.7%), *luk*D-E/*luk*PV83 (18/258, 6.9%).

Hemolysins (α, β, γ and δ) are among the important virulence factors produced by staphylococci. Hemolysins have lytic activity on a variety of host cells, resulting in death of the target cell (10). In this study, all isolates presented hemolysin genes with various combinations. Similarly, Achek *et al*. (31) found all isolates to be positive for *hld* and *hla* genes. However, in another study conducted by Azara *et al.* (32), hemolysin genes were reported in 85.3% of the isolates, either alone or in combination.

The *agr* typing has been used to determine an association between the carriage of virulence genes and the presence of the *agr* operon in clinical *S. aureus* isolates (8). In the afore mentioned study, all isolates were assigned to *agr* type I. In contrast to the findings in the present study, *agr* type I and *agr* type III were reported to be dominant *agr* allels in *S. aureus* isolates from sheep mastitis cases (31, 44).

This study provides comprehensive data related to *S. aureus* from clinical sheep mastitis in the Şanlıurfa region of Turkey, and a high prevalence of virulence-associated determinants shows a potential risk to farm animals, farmers, and consumers.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

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