Investigation of the Toxin Genes and Antibiotic Resistance in *Staphylococcus aureus* Isolates from Subclinical Mastitic Cow Milk

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ABSTRACT

Subclinical mastitis is one of the most common mastitis forms of dairy herds. Besides its economic importance, it has a serious zoonotic potential due to the spread of bacteria and toxins through milk. Among the many bacterial pathogens that may cause mastitis, *Staphylococcus aureus* is probably the most important contagious agent in the mammary gland as it causes chronic and profound infections. This microorganism produces a virulence factor and various toxins that facilitate its pathogenicity. The aim of this study was to investigate some important toxins (Panton-Valentine leucocidin, toxic shock syndrome toxin 1, exfoliative toxin), genes (*luk*-PV, *tst*-1, *eta, atb, etd*) and antibiotic resistance in *S. aureus* isolates from cows' milk with subclinical mastitis in Western Turkey. Materials for the study consisted of 31 *S. aureus* isolates obtained from 200 subclinical mastitis milk samples. Polymerase chain reaction (PCR) was used to confirm bacterial identification and to determine toxin genes. Antimicrobial resistance to eight antibiotics belonging to eight antimicrobial families was determined by the disc diffusion method. Fifteen (48.4%) isolates were found to harbor one or more toxin genes. None of the investigated isolates harbored *etb* gene, while eleven (73.3%), five (33.3%), four (26.6%) and two (13.3%) isolates had *etd*, *luk*-PV, *tst*-1, *eta* genes, respectively. A total of five toxin gene profiles were obtained. While all isolates were resistant to penicillin G, sixteen (51.6%) of them were found to be multidrug resistant. A total of 10 antibiotic resistance phenotypes were determined. The results show that there was a wide variety of *S. aureus* isolates that can lead to mastitis due to the toxin gene diversity. In addition, the high number of multi-resistant isolates to antibiotics show that there is a serious problem of antimicrobial resistance in the farms where these materials were obtained. It is thought that the presence of *S. aureus* isolates with the toxin gene in raw milk may be a potential threat to public health if they are consumed without adequate heat treatment.

Key words: Subclinical Mastitis; *Staphylococcus aureus*; Toxin gene; Antibiogram.

INTRODUCTION

Staphylococcus aureus is one of the most important contagious mastitis pathogens in cows and can cause many diseases in humans and animals (1). This microorganism is an important source of intra-mammary infection that causes serious economic losses in the dairy industry throughout the whole world (1,2). Naturally, *S. aureus* is found in the skin and mucous membranes and it is transmitted by milking machines and milking hands (1).

The pathogenic potential of *S. aureus* is based on numerous virulence factors and its ability to produce various toxins (3). Although *S. aureus* has many toxins contributing to virulence, leukocidins (Panton-Valentine Leucocidin, PVL), toxic shock syndrome toxin-1 (TSST-1) and exfo-

liative toxins (ETA, ETB, ETD) are the most important toxins in humans (3, 4). PVL, encoded by 2 genes placed on a prophage, causes leukocyte destruction and tissue necrosis (5). The presence of PVL-encoding genes in *S. aureus* has been reported to be associated with disease severity (6). Some *S. aureus* isolates produce enterotoxins and TSST-1, which may lead to staphylococcal food poisoning and human toxic shock syndrome, respectively (7). TSST-1 consists of a single-chain polypeptide with a molecular weight of approximately 22 KDA. Although the toxin belongs to the superantigen family, this toxin produced by bacteria is from a larger family of pyrogenic exotoxins (8). TSST-1 causes toxic shock syndrome in humans and animals. TSST-1 has many biological properties associated with other pyrogenic exotoxins, including IL-1, TNF-α and the ability to induce nonspecific T cell proliferation, to increase fatal endotoxin shock and induce non-specific T cell proliferation. The *tst* gene encodes the toxic shock syndrome toxin, which *S. aureus* uses to facilitate colonization (9). The staphylococci, which can produce exfoliative toxin, cause staphylococcal scalded skin syndrome and bullous impetigo. The exfoliative toxins cause bubbles in human and animal skin (10).

The common use of various antibiotics on farm animals can cause the spread of antimicrobial resistance in bacteria isolated from animals. These antibiotic resistant bacteria can pass directly to humans or indirectly through the products, which are obtained from these animals and consumed by humans without proper heat treatment (11). A number of studies on antibiotic resistance in bacteria isolated from dairy cows mastitis showed that the most common mastitis agent is *S. aureus* in Turkey (12,13,14). Penicillin and ampicillin resistance in *S. aureus* strains isolated from subclinical mastitis has been reported to be very high in Turkey (13, 14).

According to our current information, studies on staphylococcal bovine mastitis in Turkey focuses on the investigation of the staphylococcal enterotoxins and PVL genes (14). Currently, very little is known about the existence of other important toxin genes and antibiotic resistance profiles of *S. aureus* isolated from subclinical mastitis in Western Turkey. In this study, we aimed to investigate the most important toxins (toxic shock syndrome toxin 1, Panton-Valentine leukocidin, exfoliative toxin), genes (*tst*-1, *luk*-PV, *eta, etb, etd*) with polymerase chain reaction (PCR) and antibiotic resistance by disk diffusion method in *S. aureus* isolates obtained from cows' milk with subclinical mastitis.

MATERIAL AND METHODS

Clinical Examination

California Mastitis Test (CMT) was used to detect subclinical mastitis. The procedures and interpretations have been described previously (15). The CMT results were scored based on gel formation. Negative (0), weak positive (1), distinct positive (2) and strong positive (3). Positive cows were defined as having at least one quarter with CMT score of >1 (15).

Milk Samples

Milk samples were collected from the lactating cows with subclinical mastitis. Samples were taken from cows, which had not undergone any treatment for the last two weeks. A total of 380 dairy cows were examined for suspicious subclinical mastitis in 20 different farms and 6-25 samples were taken from each farm. Out of 380 cows, 200 cows were determined with subclinical mastitis and a total of 200 milk samples were taken from these mastitic cows (one milk sample with the highest CMT score was obtained from each animal). All dairy farms ranging in size from 5 to 50 animals were scaled as small and medium-sized. They all used milking machines and the age of the cows varied between 3 to 10 years.

Sample Collection

A total of 200 milk samples from quarters from 200 CMT positive cows were taken under aseptic conditions. During the collection, the following procedure was followed: teat ends were cleaned using 70% alcohol moistened swabs and allowed to dry. After discarding the first few streams, 2-5 ml of the milk samples were collected into sterile 5 ml glass flasks. Samples were refrigerated at 4°C and transported to the laboratory within 3 hours and examined as soon as possible (15).

Microbiological Examination

Milk samples were centrifuged at 3500 rpm for 5 min and the supernatant was discarded. The sediment was vortexed and a loopful were inoculated in Mueller Hinton Broth with 6.5% salt, incubated at 37°C for 18-24 hrs, grown in broth and streaked on Mannitol Salt Agar (MSA) (Merck 1.05404). After incubation at 37°C for 24 hours, the colonies from MSA plate were transferred to Tryptone soy agar (TSA)

Table 1: The antimicrobial agents used, disc contents and evaluation criteria (16)

Antimicrobial Family	Antibiotic	Disc Content (µg)	Zones (Evaluation, mm)		
			S ≥	(I)	R ≤
β Lactams	Ampicillin	10	29		28
Folate inhibitors	Trimethoprim/ sulfamethoxazole	1.25/23.75	16	11-15	10
Tetracycline	Tetracycline	30	19	15-18	14
Quinolons	Enrofloxacin	5	22		22
Penicillins	Penicillin G	10 IU	29		28
Oxazolidinones	Linezolid	30	21		21
Glycopeptides	Vancomycin	30	15		15
Macrolides	Erythromycin	15	23	14-22	13

R: Resistant, **S:** Sensitive, **I:** Intermediate

(Merck 1.05458) plate in order to observe the appearance of the characteristic colony. Then, the staphylococci were identified based on Gram's staining, pigment production and biochemical reactions: catalase activity, coagulase test (rabbit plasma), oxidase test, mannitol fermentation (15). After that, the isolates were stored in Skim Milk Powder (Fluka Analytical 70166-500G) 15%, at -20°C.

Antimicrobial Susceptibility Profiles

Antibiotic susceptibility tests were performed according to Kirby-Bauer disk diffusion method. Antibiotics tested belonged to the eight antimicrobial families used for the treatment of mastitis in the field. The antimicrobial agents used, disk contents and the evaluation criteria are shown in Table 1. The inhibition zone diameters around the disc were measured and the evaluation was made according to the previously reported methods (16). *S. aureus* ATCC 29213 strains were used as quality control in antibiotic susceptibility test.

Genotyping Identification

After the *S. aureus* was determined phenotypically by biochemical tests, identification was confirmed genotypically by PCR. In genotypic identification, the presence of the *nuc* gene encoding the thermonuclease enzyme localized on *S. aureus* chromosome was investigated in all isolates (17).

DNA Extraction

DNA extraction from *S. aureus* was performed using a commercial genomic DNA extraction kit (Fermentas, Lithuania) according to the recommendations of the manufacturer. DNA purity and quantity controls were also performed. The OD260/OD280 ratio indicated that the DNA the purity ratio was 1.8-2.0 (18).

Polymerase Chain Reaction

PCR was used for the verification of the *S. aurues* isolates and the detection of the most common *S. aureus*'s toxin genes. The DNA was amplified to investigate the presence of 5 genes that can contribute in different ways to *S. aureus* pathogenicity in humans. In this study, genes encoding leucocidins (*luk*-PV), genes related to host invasion (*eta, etb, etd*), and factors that have the potential to interfere with host defense mechanisms (*tst*) were analyzed using primers and protocols described in literature and listed in Table 2 (17, 19, 20, 21).

PCR, for each sample was carried out in a volume of 50 µl, final concentration was 10x Taq enzyme buffer solution 1x, 25 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), 2 µl of each DNA. The tubes were loaded in the thermal cycler. The DNA was amplified using the following protocol: an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation (94°C for 30s), annealing (54°C for *nuc*, *eta* and *etd*, 55°C *etb*, *tst*-1 and *luk*-PV, for 30s) and extension (72°C for 1 min), with a single final extension of 7 min at 72°C. The amplified PCR fragments were visualized 100 V for 45 min. on 2% agarose gel stained with safe view (5µl/100ml; ABM, Richmond, Canada) and visualized by UV transilluminator (*Vilber Lourmat*, Collégien, France) and photographed. A 100 bp DNA ladder (Fermentas, Lithuania) was used in each gel.

Sequence Analysis

In the study, as a positive control for the toxin genes examined by the polymerase chain reaction, sequenced field isolates were used, which were found to carry these genes. For this purpose, the positive amplicons were sent to a specialized company (Macrogen, Amsterdam, Netherlands) for sequence analysis. Sequence analysis was performed after the purification procedure. Following this, these sequences were compared with the gene bank. The nucleotide-nucleotide

Table 2: Primers used in the study

Target Gene	The toxin encoded by the target gene	Sequence (5'→3')	Tm	Product Size (bp)	Reference
nuc	Thermonuclease	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	55	279	17
eta	Exfoliative toxin A	CTATTTACTGTAGGAGCTAG ATTTATTTGATGCTCTCTAT	55	741	19
etb	Exfoliative toxin B	ATACACACATTACGGATAAT CAAAGTGTCTCCAAAAGTAT	57	629	19
etd	Exfoliative toxin D	AACTATCATGTATCAAGG CAGAATTTCCCGACTCAG	55	376	19
tst-1	Toxic shock syndrome toxin 1	AGCATCTACAAACGATAATAT CATTGTTATTTTCCAATAAAC	57	481	20
luk-PV	Panton-Valentine Leukocidin	ATCATTAGGTAAAATGTCTGGACATGATCCA GCATCAASTGTATTGGATAGCAAAAGC	57	433	21

Tm: Melting Temperature

Table 3: Number and ratio of toxin genes carried by *S. aureus* isolates

No.	*eta*	*etb*	*etd*	*tst*-1	*luk*-PV
1	–	–	+	–	–
2	–	–	+	–	–
3	–	–	+	–	–
4	–	–	+	–	–
5	–	–	+	–	–
6	–	–	+	–	+
7	–	–	+	–	+
8	–	–	+	–	+
9	–	–	+	–	–
10	–	–	+	–	+
11	+	–	–	+	–
12	+	–	–	+	–
13	–	–	–	+	–
14	–	–	–	+	+
15	–	–	+	–	–
Total	2	0 (0.0)	11	4	5

BLAST program was used for this purpose. The highest homology type was considered the same as the type of sequence detected.

RESULTS

Isolation and Identification

All colonies that were grown in yellow and pink color on MSA were Gram stained. Sixty-five isolates (32.5%, 65/200) which were catalase positive and oxidase negative were identified as staphylococci species. The presence of coagulase enzyme was considered as criteria for pathogenicity of *S. aureus* (15). For that reason, the coagulase test was performed for all of the staphylococci isolates. Thirty one (15.5%, 31/200) isolates were coagulase positive and therefore these isolates were identified as *S. aureus* and 12 farms (60%) from which the material was taken were considered as positive.

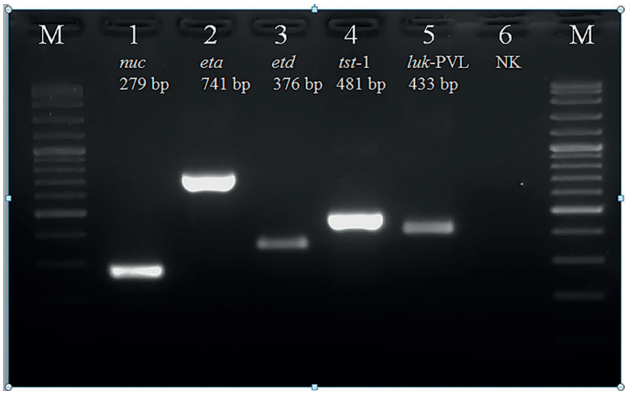

Figure 1: Gel electrophoresis of toxin genes. **M:** 100 bp DNA Ladder **1.** *nuc* gene positive *S. aureus* ATCC 29213 strain **2.** *eta* gene positive sequenced site isolate **3.** *etd* gene positive sequenced site isolate **4.** *tst*-1 gene positive sequenced site isolate **5.** *luk*-PV gene positive sequenced site isolate **6.** Negative control (Master mix without DNA).

Genotypic Identification

After PCR was performed with the *nuc* gene-specific primer, 277 bp of PCR product was obtained in all 31 isolates and

Table 4: Distribution of toxin genes of *S. aureus* isolates

Genotype	Toxin genes	Isolate Number (%)
1	*etd*	7 (46.6)
2	*tst*-1	1 (6.6)
3	*etd* + *luk*-PV	4 (26.6)
4	*eta* + *tst*-1	2 (13.3)
5	*tst*-1 + *luk*-PV	1 (6.6)
TOTAL		15

Table 5: Antibiotic resistance rates of isolates of *S. aureus*

Antibiotic	Suscebtible S (%)	Intermediate I (%)	Resistant R (%)
Penicillin G	0 (0.0)	0 (0.0)	31 (100.0)
Ampicillin	13 (41.9)	0 (0.0)	18 (58.1)
Trimethoprim/ Sulphametaxazole	11 (35.5)	4 (12.9)	16 (51.6)
Tetracycline	13 (41.9)	7 (22.6)	11 (35.5)
Erythromycin	12 (38.7)	10 (32.2)	9 (29.1)
Enrofloxacin	31 (100.0)	0 (0.0)	0 (0.0)
Linezolid	31 (100.0)	0 (0.0)	0 (0.0)
Vancomycin	31 (100.0)	0 (0.0)	0 (0.0)

*Total Isolate Number: n=31

the isolates were confirmed to be *S. aureus*. Later, the toxin genes of these isolates were analyzed by PCR and the antibiotic resistance was determined by the disk diffusion method.

Toxin Genes

In this study, the presence of five important toxin genes (*eta*, *etb*, *etd*, *tst*-1, *luk*-PV) were determined by PCR. The study showed that fifteen (48.4%, 15/31) of isolates had at least one of these toxin genes. It was determined that eleven (73.3%, 11/15) of the isolates carrying toxin gene were *etd*, five (33.3%, 5/15) were *luk*-PV, four (26,6%, 4/15) were *tst*-1, two (13.3%, 2/15) were *eta* genes. None of the isolates carried the *etb* gene (Table 3, Figure 1). Seven (46.6%, 7/15) of the isolates had only *etd* and one (6.6%, 1/15) *tst*-1 gene, four (26.6%, 4/15) of both *etd* and *luk*-PV, two (13.3%, 2/15) of both *eta* and *tst*-1, one (6.6%, 1/15) of both *tst*-1 and *luk*-PV genes together (Table 4).

Antibiotic Resistance

The resistance profiles of eight antibiotics from eight antimicrobial families were studied. Out of 31 *S. aureus* strains, thirty-one (100.0%, 31/31) were found as resistant to penicillin G, eighteen (58.1%, 18/31) to ampicillin, sixteen (51.6%, 16/31) to trimethoprim/sulfamethoxazole, eleven (35.5%, 11/31) to tetracycline, nine (29.1%, 9/31) to erythromycin. All isolates were also susceptible to enrofloxacin, linezolid and vancomycin (Table 5). The bacteria with three or more antimicrobial resistance were considered as multi-resistant (22). While all isolates were resistant to penicillin G, sixteen (51.6%) of them were multidrug resistant. A total of 10 antibiotic resistance phenotypes were determined. It was determined that six (19.4%, 6/31) and ten (32.3%, 10/31) of the isolates were resistant to 3 and 4 antimicrobial families, respectively (Table 6).

Table 6: Antibiotic resistance patterns in *S. aureus* isolated from subclinical mastitis

Number	Antibiotics resistance patterns	Number (%) of resistant isolates All isolates (n=31)
1	P	3 (9.6%)
2	P, TE	5 (16.0%)
3	P, E	1 (3.2%)
4	P, SXT	3 (9.6%)
5	P, AMP	3 (9.6%)
6	P, SXT, E	1 (3.2%)
7	P, E, AMP	3 (9.6%)
8	P, AMP, SXT	2 (6.4%)
9	P, TE, SXT, AMP	6 (19.2%)
10	P, E, SXT, AMP	4 (12.8%)

AMP: Ampicillin, **SXT:** Trimethoprim/Sulfametaxazole, **TE:** Tetracycline, **P:** Penicillin G, **E:** Erythromycin

DISCUSSION

Economic losses of farmers are increasing due to decreasing milk production and increasing treatment costs. *Staphylococcus* species, particularly *S. aureus*, is considered to be one of the most important aetiological agents of subclinical mastitis because *S. aureus* is a contagious pathogen that may have zoonotic effects. In a study conducted in Sweden, 31.0% *S. aureus* and 27.0% coagulase negative staphylococci (CNS) were isolated from 226 dairy farms as a result of the cultural examination of 583 milk samples from subclinical mastitic cows (23). In another study conducted in Ireland, samples from the infected udder quarter were evaluated from 285 bovine animals from 15 farms and 21.0% *S. aureus* and 9%

CNS were reported (24). In a study from Turkey, 49.7% of 235 cow milk samples with subclinical mastitis were found with *Staphylococcus* spp. isolated. Of these 117 isolates, 63.3% (74 isolate) and 36.7% (43 isolate) were *S. aureus* and CNS, respectively (25). In the present study, 32.5% (65 isolate) *Staphylococcus* spp. were isolated from 200 cow milk samples from 20 farms with subclinical mastitis. From these samples, 31 (47.7%) of them were identified as *S. aureus* and 34 (52.3%) as CNS. All 31 strains with coagulase activity were identified as *S. aureus* by species-specific PCR. The prevalence of *S. aureus* may be related to geographical regions, breed of cow, lactation stage, and udder hygiene. Contagious microorganisms especially *S. aureus* are usually found on the teat surface. Contamination usually occurs during milking.

S. aureus has been recognized as a contagious pathogen with zoonotic effects (15). It has the potential to produce a number of extracellular toxins and virulence factors. In this study, the presence of genes relatively less studied but important toxin genes were examined of *S. aureus* causing mastitis. Exfoliative toxins cause different lesions on the skin surface. The *eta* gene is encoded by the *etb* plasmid (10). Kot *et al.* (2016) reported that the *eta* and *etd* genes, encoding exfoliative toxins, were present in 5.6% and 8.9% of isolates, respectively in Poland (26). Schmidt *et al.* (2017) reported that none of the bovine isolates tested positive for the exfoliative toxin genes *eta, etb*, or *etd* in South Africa (27). Monistero *et al.* (2018) were reported that *S. aureus* isolates isolated from cattle mastitis from eight countries have not been able to detect the presence of the *etb* gene and only one South African isolate was positive *eta* gene (4). There was no *etb* gene in our isolates, however *eta* gene was found in 2 of the toxin gene bearing 15 isolates. Among our isolates, the most common gene was found to be *etd*. The results in our study demonstrated that *eta* and *etb* are rarely present in *S. aureus* isolated from cattle with mastitis, which is in line with previous studies conducted in different countries (4, 26). Toxic shock syndrome toxin belongs to the superantigen family, which includes large pyrogenic exotoxins (9,10). The *tst* gene was found in 2.4% of the isolates from Poland (26), 37% of the isolates from Argentina, 23% of the isolates from Germany, 16% of the isolates from Tunesia and 6% of the isolates from Italy (4). In our study, the *tst* gene was present in 4 (26.6%) of 15 virulence gene bearing isolates. Although there are studies showing that leukocidins have an important role in the pathogenesis of subclinical cow mastitis (28, 29), some researchers reported that PVL do not play a very important role in other studies (14, 26). The PVL gene was identified in more than 50% of isolates from bovine mastitis in Italy (28). In China, the prevalence was reported to be 41.5% in the bovine isolates (29). However, other studies could not detect PVL gene in *S. aureus* isolates from bovine milk (30, 31). In our study, the PVL gene was present in 5 (33.3%) of 15 virulence gene isolates.

The resistance of *S. aureus* to antibiotics is a major global problem. In dairy farms, antibiotics such as penicillin, cephalosporin and tetracycline are used for the treatment of mastitis (32). In this study, the antimicrobial resistance profiles of *S. aureus* were determined and high levels of resistance to penicillin (100%) followed by ampicillin (58.1%) were detected. Penicillin and ampicillin resistance are common in mastitis cases caused by *S. aureus* in Turkey, but may vary greatly between geographic regions (13, 33). Similar results were obtained by Khakpoor *et al.* (34) who reported that all *S. aureus* isolates were resistant to penicillin and ampicillin. Previous studies reported generally low rates (13, 35) of resistance to trimethoprim-sulfamethoxazole for *S. aureus*. However, Alekish *et al.* (2013) reported 87.4% of the isolates were resistant against trimethoprim/sulphamethoxazole (35). The occurrence of 35.5% resistance to tetracycline in this study is much higher than that of 13.0% as reported by Liu *et al.* (36) and lower as compared to 54.0% resistance reported by Mekonnen *et al.* (37). The occurrence of 29.1% resistance to erythromycin in this study is much higher than that of 4.0% as reported by Mekonnen *et al.* (37) and lower as compared to 46.3% resistance reported by Lui *et al.* (36). The variability of the resistance may be partly due to how often a drug is used in the study area.

The present study showed that 9.6% of isolates had resistance to a single antibiotic, 38.7% of the isolates to 2 antibiotics, and 51.6% of the isolates to several antibiotics. The result of this study was similar from those obtained by Chaalal *et al.* (38) for single and multiple resistance (5.3% and 83.9%). Multiple antibiotic resistance can be attributed to the use of random antibacterial drugs without antimicrobial susceptibility testing. Such multiple antimicrobial resistant microorganisms can cause serious health hazards for humans as well as animals. Recently, the rate of antimicrobial resistance from bovine mastitis has been reported to increase in *S. aureus* (39).

In this study, it was found that multi-drug resistant *S. aureus* isolates were common in dairy farms. Determining that *S. aureus* isolates from mastitic cows had multiple resistance to commonly used antibiotics, ensures that correct antibiotic use is very important in the treatment and control of infections caused by *S. aureus*. In future studies, it is recommended that resistance determinants for multidrug-resistant microorganisms be determined using molecular methods. The results show that there are a wide variety of *S. aureus* isolates that can lead to mastitis due to the toxin gene diversity and the high number of multi-resistant isolates to antibiotics shows that there is a serious problem of antimicrobial resistance in the farms from where these materials were taken. It is considered that the presence of *S. aureus* isolates with the toxin gene in raw milk may be a potential threat to public health if they are consumed without adequate heat treatment.

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