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Original Article

Enterotoxin gene profile of *Staphylococcus aureus* isolates recovered from bovine milk produced in central Ethiopia

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Abstract
Introduction: Staphylococcal food intoxication is dependent on the production of enterotoxins, the single most important virulence factors. Various studies conducted in Ethiopia have depicted the prevalence of *S. aureus* in bovine milk. However, there is no published data regarding the enterotoxin gene profile of *S. aureus* isolates in Ethiopia. The aim of this study was, therefore, to evaluate enterotoxin gene carriage profile of *S. aureus* isolates recovered from bovine milk samples from central Ethiopia.
Methodology: In this study, 109 *S. aureus* isolates recovered from bovine milk were analyzed for carriage of the classical enterotoxin genes. Genomic DNA extraction was performed using a commercially available kit. Two sets of multiplex polymerase chain reaction (PCR) assays were used to detect the five classical enterotoxin-coding genes and the toxic shock syndrome toxin gene.
Results: At least one type of *S. aureus* enterotoxin gene (SE) was carried in 73 (66.9%) of the isolates. The most frequently encountered gene was *sea* (40; 36.7%) followed by *seb* (19; 17.4%), *see* (18; 16.5%), *tst* (16; 14.7%), *sec-1* (12; 11.01%), and *sed* (7; 6.4%). Of the 73 *S. aureus* isolates harboring at least one of the enterotoxin genes, 26 (35.6%) strains harbored more than one enterotoxin gene.
Conclusions: More than half of the *S. aureus* isolates harbored at least one of the enterotoxin coding genes, indicating milk specimens contaminated by *S. aureus* could have a high chance of causing food intoxication.

Key words: enterotoxins; dairy; food intoxication; Ethiopia.

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Introduction
Staphylococcus aureus is known to be involved in causing food poisoning outbreaks and has also been reported in association with food products including milk from different parts of the world [1-6]. Staphylococcal food poisoning is characterized by an acute onset of nausea, vomiting, abdominal cramps, and diarrhea [7]. Staphylococcal food intoxication is dependent on a single type of virulence factor that is the production of enterotoxins by certain *S. aureus* strains [7]. A large variety of enterotoxins are produced by *S. aureus* strains. The available literature shows that their nomenclature (A, B, C, D, E, G, H, I, J, K, L, M, N, O, P, Q, R, and U) is dynamic, as more and more novel staphylococcal enterotoxins (SEs) are being identified [8]. The first five (A to E) classical enterotoxins are known to cause 95% of the food poisoning globally [9,10]. There is a strong association between the ability of *S. aureus* strains to produce one or more of the SEs and the occurrence of staphylococcal food poisoning [11].

Milk is a medium on which *S. aureus* growth as well as enterotoxin production by this pathogen is well supported. It is obvious that pasteurizing raw milk would eliminate *S. aureus* from raw milk; however, once the pathogens have produced the SEs, the toxins will remain stable even after pasteurization [12]. Researchers have shown that SEs are highly resistant to heat treatment; a good example is *sea*, which retained its biological activity even after exposure to as high temperature as 121°C for 28 minutes [13].

A very small amount of SEs, ranging from 20 ng to < 1 μg, is needed to cause a typical symptom of staphylococcal food poisoning [14]. Humans predominantly encounter enterotoxins from consumption of milk and dairy products [15].

Investigating the capability of strains of *S. aureus* to produce enterotoxins is, therefore, important to prevent the public health risk associated with consumption of milk and dairy products.

Various studies have been conducted in Ethiopia to depict the prevalence of *S. aureus* in bovine milk. Most of these studies were done in association with mastitis cases and showed the importance of this pathogen in causing mastitis [16-18]. Data regarding the ability of *S. aureus* isolates to produce enterotoxins and also carriage of enterotoxin coding genes is not available in Ethiopia. In addition, the potential significance of milk and milk products as a likely source of staphylococcal food intoxication is lacking in the Ethiopian context. The aim of this study was, therefore, to evaluate the enterotoxin gene profiles of *S. aureus* isolates recovered from bovine milk samples from central Ethiopia.

Methodology

Bacterial isolates

One hundred and nine polymerase chain reaction (PCR) and biochemically-confirmed *S. aureus* isolates that were recovered from on-farm pooled and combined bulk tank milk samples from central Ethiopia were used in this study. The isolates were obtained from milk samples collected from four different geographical areas of central Ethiopia, namely Selale (n = 60), Asela (n = 13), Debre-Zeit (n = 24), and Addis Ababa (n=12), over a period of nine months in 2011 and 2012.

DNA extraction

Genomic DNA extraction was performed using commercially available kit (Qiagen GmbH, D-40720, Hilden, Germany). Bacterial DNA was extracted according to the protocol provided by the manufacturer and Infectious Disease Molecular Epidemiology Laboratory (IDMEL) of College of Veterinary Medicine, Ohio State University. Briefly, bacterial isolates were sub-cultured on tryptic soy agar (TSA). Colonies were harvested and suspended using molecular grade water in a microcentrifuge tube and centrifuged for 10 minutes at 10,000 rpm. The supernatants were discarded and the bacterial pellets were resuspended in 180 µL of enzymatic lysis buffer. DNA was eluted in 100 µL of molecular grade water and was stored at an appropriate temperature for further analysis (4°C).

Amplification of SE genes

Two sets of multiplex PCR assays were used to detect the enterotoxin coding genes. Set A included *sea*, *sec-1*, and *tst*-1 genes (toxic shock toxin-1), and set B included *see*, *sed*, and *seb*. The primers used to amplify each of the genes are shown in Table 1. PCR reaction was performed in a final volume of 25 µL (10 µmol pair of primers for each gene = 0.75 µL; 19.5 µL of RNase-free water and 1 µL of template DNA). Ready-to-go PCR beads (PuReTaq Ready-To-Go PCR Beads; GE Healthcare UK Limited, Buckinghamshire, UK) were also used to amplify the enterotoxin genes. Amplification was carried out using a thermocycler (PTC-100 programmable Thermal Controller MJ Research Inc., Foster city, CA, USA). The PCR running conditions were as follows: initial denaturation at 95°C for 10 minutes, denaturation at 94°C for 2 minutes, annealing at 55°C for 2 minutes, extension at 72°C for 1 minute, and a final extension at 72°C for 1 minute. The number of running cycles was set to be 45. The amplicons were separated by a 1% agarose gel. Established positive controls that were available at IDMEL as culture collections were used for each of the target SE genes (STO3050 for *sea*, STO3048 for *sec-1*, STO3047 for *seb*, STO3049 for *sed*, STO3051 for *see*, and an internal control strain for *tst*-1 genes).

Toxin gene profile data analysis

Gel images were analyzed using a Gel Reader (Gel Doc 2000, BioRad, Hercules, CA, USA), and the

Table 1. List of primers used to amplify staphylococcal enterotoxin genes (adapted from previous authors [31]).

SE gene	Primers	Sequence (5' to 3')	Amplicon size (bp)	Location within gene	Reference
sea	SEA-F	TTGGAAACGGTTAAAACGAA	120	490–509	[31]
	SEA-R	GAACCTTCCCATCAAAAACA		591–610	
seb	SEB-F	TCGCATCAAACTGACAAACG	478	634–653	
	SEB-R	GCAGGTACTCTATAAGTGCC		1091–1110	
sec-1	SEC-F	GACATAAAAGCTAGGAATTT	257	676–695	
	SEC-R	AAATCGGATTAACATTATCC		913–932	
sed	SED-F	CTAGTTTGGTAATATCTCCT	317	354–373	
	SED-R	TAATGCTATATCTTATAGGG		652–671	
see	SEE-F	TAGATAAAGTTAAAACAAGC	170	491–510	
	SEE-R	TAACTTACCGTGGACCCTTC		640–659	
tst-1	TST-F	ATGGCAGCATCAGCTTGATA	350	251–270	
	TST-R	TTTCCAATAACCACCCGTTT		581–600	

presence of bands with the expected band size for each enterotoxin gene was determined. These values were used to calculate frequency distribution. The possible association between the geographical location of the origin of the isolate and the SE status of the isolates was done using the Chi-square test (SPSS version 20).

Results and discussion

Toxin gene profile of S. aureus isolates

There has been no published information regarding SEs in the Ethiopian context. This is the first study reporting *se* gene detection from *S. aureus* isolated from bovine milk. The current study showed that among the 109 *S. aureus* isolates investigated, at least one SE gene allele was present in 73 (66.9%) of the isolates. Of these 73 isolates, 26 (35.6%) had more than one enterotoxin gene encoding the different enterotoxins with or without the *tst* gene. This result is comparable with that of a study conducted in Brazil where 68.4% of *S. aureus* isolates were reported to be positive for at least one of the genes encoding the enterotoxins [19]. In the present study, the most frequently encountered SE gene was *sea* (40/109; 36.7%) followed by *seb* (19/109; 17.4%), *see* (18/109; 16.5%), *tst* (16/109; 14.7%), *sec-1* (12/109; 11.01%), and *sed* (7/109; 6.4%). In line with the present study, Rall *et al*. [19] indicated that *sea* (41%) was the most frequent enterotoxin gene prevalent followed by *sec* (20.5%), *sed* (12.8%), *seb* (7.7%), and *see* (5.1%). This study in Brazil also reported the prevalence of enterotoxin genes other than the classical ones, with *seg* (28.2%) being the most predominant one followed by *sei* (25.6%), *sej*, and *seh* (7.7%). In the current study, however, an attempt to detect enterotoxin genes other than the classical (A to E and *tst*) ones was not done. The potential role of SEs other than the classical enterotoxins in staphylococcal food poisoning is yet to be determined [20]. In spite of this notion, some reports indicated that the newly discovered SEs, including SEH [21], SEG, and SEI [22] have a potential to induce gastroenteric syndromes.

In addition, a study conducted in Italy also showed that 67% of the *S. aureus* isolates recovered from

Table 2. Distribution of enterotoxin genes and *tst*-1 gene in the four regions Asela, Selale, Debre-Zeit, and Addis Ababa.

Enterotoxin genes	Frequency of enterotoxin genes in each region				Total
	Selale	Asela	Debre-Zeit	Addis Ababa	
	No. of isolates (%) n = 60	No. of isolates (%) n = 13	No. of isolates (%) n = 24	No. of isolates (%) n = 12	No. of isolates (%) n = 109
sea	24 (40)	5 (38.5)	6 (25)	5 (41.7)	40 (36.7)
seb	10 (16.7)	4 (30.8)	3 (12.5)	2 (16.7)	19 (17.4)
sec-1	9 (15)	1 (7.8)	2 (8.3)	0 (0)	12 (11.01)
sed	3 (5)	3 (23.1)	0 (0)	1 (8.3)	7 (6.4)
see	9 (15)	3 (23.1)	5 (20.8)	1 (8.3)	18 (16.5)
tst-1	9 (15)	2 (15.4)	1 (4.2)	4 (33.3)	16 (14.7)

Table 3. Genotypic profile of *S. aureus* isolated from raw bovine milk based on enterotoxin (A-E) and toxic shock syndrome toxin-1 gene distribution.

Genotype profile	Frequency of isolates (n = 109)	Percentage
a	17	15.6
b	8	7.3
e	12	11
tst	10	9.2
a + b	8	7.3
a + c	6	5.5
a + d	2	1.8
a + e	1	0.92
a + b + c	1	0.92
a + c + e	1	0.92
e + d + b	1	0.92
c + e + tst	1	0.92
e + d + tst	1	0.92
a + b + d + tst	1	0.92
a + c + d + tst	2	1.8
a + c + e + tst	1	0.92

different dairy products were found to be positive for one or more of the SE genes, with *sea* and *sed* being the predominant ones [23].

Distribution of SE genes among different geographical areas

The isolates for the current study were obtained from bovine milk representing four geographical locations in the central highlands of Ethiopia. The regions are known to be the major milk shed areas in central Ethiopia. The distribution of enterotoxin genes of *S. aureus* isolates from these regions is shown in Table 2.

Previous studies demonstrated that there is a geographical difference in the distribution of superantigens (SAgs) producing strains of *S. aureus* that are known to cause mastitis [24]. Comparing the distribution of SE genes within each sampling region, enterotoxin gene *sea* still remained predominant and ubiquitous in all the geographic locations, ranging in prevalence between 25% and 41.7%. A higher frequency of *seb* genes was observed among isolates from the Asela area (Table 2).

In the current study, it was found that 5.5% of the isolates had a combination of $\geq$ 2 SEs (*a*, *b*, *c*, *d*, *e*) with the *tst*-1 gene (Table 3). A study in New York showed that *S. aureus* strains producing SED alone or in combination with *S. aureus* with SEC and TSST-1 accounted for 22% of the isolates [25]. In Norway, a previous study also indicated that 58% of *S. aureus* isolates expressed SAgs and that the production of SEC and TSST-1 predominated [26]. In addition, some reports suggested that *S. aureus* strains that express SEC and TSST-1 in combination cause severe clinical mastitis that is unresponsive to treatment [27,28], while others still failed to find correlation between SAgs production and clinical manifestation of mastitis [24,26]. In the present study, four isolates (3.7%) that had a combination of *sec*-*tst*-1 genes were detected. There is a notion that SAgs might facilitate immunosuppression in cattle, thereby contributing to chronic intramammary infection; yet, some researchers still suggest that no clear-cut evidence of association between a specific SE-producing *S. aureus* strain and manifestation of subclinical mastitis exists [29]. The clinical manifestation of mastitis in Ethiopian cows with this combination of enterotoxin genes (combination of ≥ 2 SEs [*a*, *b*, *c*, *d*, *e*] with *tst*) needs to be investigated. Although there is a lack of sufficient data, the high prevalence of mastitis caused by *S. aureus* [16,18,30] combined with other factors such as limited veterinary service, poor milking hygiene, and little access to refrigerated milk prior to consumption would suggest that milk and milk products could significantly be associated with staphylococcal food poisoning in Ethiopia. The presence of enterotoxin genes in *S. aureus* isolates may not necessarily indicate that such isolates can produce the toxin that leads to food intoxication, and hence it is useful to assess the expression of the genes into their respective protein toxins. The occurrence of *S. aureus* strains with multiple enterotoxin genes, however, presents a threat to public health with respect to consumption of milk and milk products.

Conclusions

The results of this study showed that more than half of the *S. aureus* isolates harbored at least one of the enterotoxin coding genes, with *sea* being dominant, which pose a public health threat to consumers. The ability of these isolates to produce the respective active toxins in milk, however, needs to be further investigated. To the best of our knowledge, this report depicting the presence of the variety of enterotoxin coding genes in *S. aureus* isolates is the first in its kind from Ethiopia.

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