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Molecular detection of enterotoxin-producing *Staphylococcus aureus* isolates from sheep in the Sistan region southeast of Iran

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Abstract

Staphylococcus aureus (*S. aureus*) infections in livestock and humans pose serious health problems. Therefore, identifying the virulence genes of this bacterium and studying their characteristics are of great importance. The main objective of this study was to investigate the frequency of virulence (enterotoxin) genes of *S. aureus* isolated from sheep noses in the Sistan region. In this study, 100 isolates of *S. aureus* were collected. After genomic extraction of the identified isolates, a multiplex PCR reaction was performed for sea, *seb, sec, see, tsst, and pvl* genes using specific primers. In total, 49 isolates of *S. aureus* isolated contained one or more enterotoxin genes. The most abundant gene was *tsst* (37%), followed by *sec* (23%), *seb* (20%), and *sea* (2%). In general, it was found that the presence of *S. aureus* in sub-clinical animal isolates, especially enterotoxigenic strains, can be a potential health hazard.

Introduction

With the expansion of agricultural and livestock activities, the importance of animal health and disease prevention, especially in rural areas and developing countries, is becoming increasingly evident. In this context, bacteria have been strongly considered as a motivator for research in animal and human health. *Staphylococcus aureus* is an important bacterium that is mainly found in animals and humans and can lead to the development of various diseases. Among the important factors of interest in these bacteria are virulence genes or enterotoxins, which play an important role in developing clinical symptoms of multiple diseases (1, 2). According to a report by Casman and colleagues et al in 1967, the variation rate in the enterotoxigenic power of *Staphylococcus* strains

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isolated from clinical sources was 47%, while for sub-clinical strains it was reported to be about 31% (1).

Staphylococcus is a Gram-positive, facultative anaerobic coccus belonging to the family *Micrococcaceae*. The most important species in the genus *Staphylococcus*, such as *S. aureus*, *S. epidermidis*, *S. saprophyticus*, and *S. lugdunensis*, are considered medically and veterinary important. *Staphylococcus aureus* is naturally found in the mucous membranes of the nose, respiratory tract, and skin of humans and animals (2, 3). Among the enterotoxins of *S. aureus*, type A enterotoxin is the most commonly reported enterotoxin. The gene for this enterotoxin is carried by bacteriophages, in contrast, the genes for enterotoxins B (*seb*) and C (*sec*) are located on the chromosome, which can contribute to the greater spread and prevalence of this enterotoxin compared to other enterotoxins (5). Enterotoxin B is also important because it can be absorbed through inhalation and has bioterrorism applications. Enterotoxin C causes food poisoning by horizontally entering into pathogenicity islands, and the *see* gene that encodes enterotoxin E is located on the chromosome of *S. aureus* and is involved in food poisoning in dairy products such as raw milk and cheese, raw meat, vegetables, and sweets (6). Rural areas and livestock farms, especially in areas with of specific climatic influences, provide suitable infrastructure for spreading bacteria and diseases. Sistan, located in Iran, is known as an active livestock farming region. Therefore, the examination and identification of virulence (enterotoxin) genes present in *S. aureus* isolated from the noses of sheep in this region can not only help veterinarians to better understand the status of animal health in this region, but also the information obtained from this research can be used as a basis for prevention and control measures for these bacteria and related diseases in animals and humans.

Materials and methods

Study Area

Sistan and Baluchestan Province is located in southeastern Iran, and Sistan is located in the northern part of the province between 25 degrees and 3 minutes to 31 degrees and 27 minutes north latitude from the equator and 58 degrees and 50 minutes to 63 degrees and 21 minutes east longitude from the Greenwich Meridian (Figure 1). It has a semi-desert climate. The north of the province, rising from the alluvial deposits of the Helmand River, is home to the largest freshwater lake in the world in times of abundance (https://www.usb.ac.ir/en/About-USB/About-Sistan-and-Baluchestan-Province).

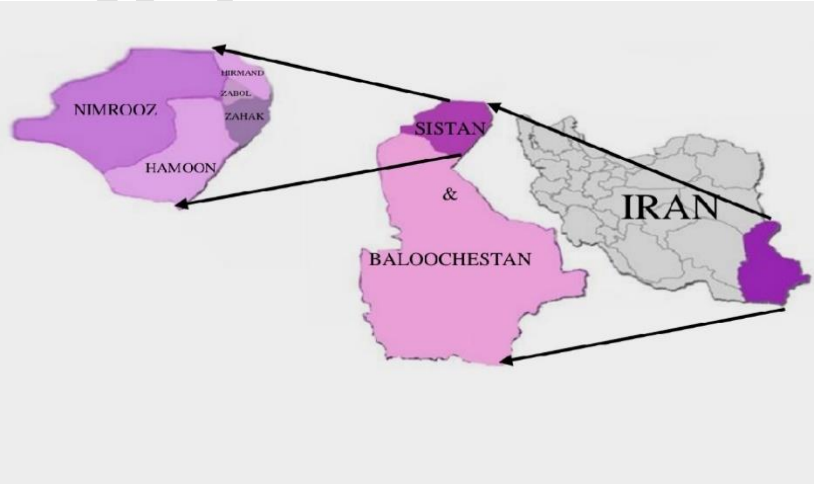

Fig. 1. Frequency of the genes under study in a hundred samples

Sample Collection

First, 4 mL of nutrient broth (Merck, Germany) was prepared in a Falcon 15 for sampling. A total of 300 samples were collected from the noses of sheep using a sterile swab in nutrient broth. The freshly collected sample in the nutrient broth was immediately transferred to the microbiology laboratory at Zabol University's Faculty of Veterinary Medicine.

Isolation of Staphylococcus aureus isolates

In this study, 100 *S. aureus* isolates were isolated from 300 nasal samples of sheep in the Sistan region cultured on mannitol salt agar (Merck, Germany). The macroscopic characteristics of bacterial colonies such as size, shape, clarity, and color were considered, and the bacteria were isolated using more accurate biochemical tests such as positive coagulase, positive DNase, positive hemolysis, positive mannitol fermentation, and positive maltose fermentation (2, 5).

DNA Extraction

In this study, the boiling method was used for the extraction of genomic DNA. First, a few pure colonies of *S. aureus* were inoculated into a tube containing 5 mL of BHI broth (Brain Heart Infusion) (Merck, Germany) and incubated at 37 °C for 12 to 18 h. Then, 1 mL of the above medium was poured into 1.5 mL sterile tubes and centrifuged at 3000 rpm (6, 7). In the next step, the supernatant of the microtubes was completely drained 200 µL of sterile distilled water was added, and the mixture was incubated at 100 °C in a thermal block (Eppendorf, Germany) for 10 min. After this step, the tubes containing the lysed cells were centrifuged at 13000 rpm, the supernatant was transferred to a 1.5 mL tube, and the quantity and quality of the extracted DNA were evaluated by NanoDrop 2000c (Thermo Scientific, USA). The extracted DNA was stored at -20 °C for future use.

Primer Preparation

In this technique, six pairs of primers (Table 1) were prepared to amplify each of the enterotoxin genes present in the genome of *S. aureus* from the Tehran Pioneer Biotechnology Company and were made available to the laboratory for the work. The primers were lyophilized and before use, the preparation steps of each of them were performed according to the manufacturer's instructions as follows.

Multiplex PCR reaction

A multiplex PCR assay for the identification of six enterotoxin-encoding genes (Table 1) was performed in a Thermo Scientific Eppendorf thermal cycler. To perform this reaction, 5.5 µL of double-distilled water, 12.5 µL of master mix, 0.2 µM of each of the corresponding primer pairs, and 5 µL of template DNA were mixed, and the final volume was brought to 25 µL with double-distilled water. The mixture was then placed in a thermal cycler (Eppendorf, Germany). The PCR product was then electrophoresed on a 1.5% agarose gel with SYBR Safe staining (E0203, Labnet) and visualized using Gel Documentation (Vilber Lourmat, France). The positive rate of *Staphylococcus* spp. DNA using the *sea*, *seb*, *sec*, and *tsst* genes was 2%, 20%, 23%, and 37%, respectively.

Phylogenetic analysis and construction of phylogenetic tree

The sequences were uploaded to the National Center for Biotechnology Information (NCBI) to search for the most similar reference sequences. In addition, the COI positions of country of origin information were identified using NCBI BLAST (accession numbers OQ550021, OP924107, OQ550022, and 2651850). For phylogenetic analysis, all available COI sequences of Staphylococcus species were used from the NCBI database (8). The alignment was manually adjusted using the Clustal W program to remove any associated errors before exporting as MEGA 11 files. All obtained nucleotide sequences were entered into the NCBI database and given accession numbers (Table 1).

Table 1. Primers used in this study.

Gene name		Sequence 5'---------- 3'	PCR product (Base pair)
sea	F	GAAAAAAGTCTGAATTGCAGGGAACA	**561**
	R	CAAATAAATCGTAATTAACCGAAGGTTC	
seb	F	ATTCTATTAAGGACACTAAGTTAGGGA	**404**
	R	ATCCCGTTTCATAAGGCGAGT	
sec	F	GTAAAGTTACAGGTGGCAAAACTTG	**297**
	R	CATATCATACCAAAAAGTATTGCCGT	
see	F	CAAAGAAATGCTTTAAGCAATCTTAGG	**482**
	R	CACCTTACCGCCAAAGCTG	
tsst	F	TTCACTATTTGTAAAAGTGTCAGACCCACT	**180**
	R	TACTAATGAATTTTTTTATCGTAAGCCCTT	
pvl	F	GGAAACATTTATTCTGGCTATAC	**505**
	R	CTGGATTGAAGTTACCTCTGG	

Results

Multiplex PCR

A total of 300 samples were collected from the noses of sheep using a sterile swab. One hundred positive samples of *Staphylococcus aureus* were obtained after conducting biochemical tests. The frequency of different patterns of the studied genes in the present study is shown in Table 2. Of the total 100 sub-clinical isolates, the number of isolates containing the *tsst* and *sec* genes was relatively more common (37% and 23%); moreover, the *see* and *pvl* genes were not observed in any of the clinical samples.

Among the 100 isolates, 37 isolates contained the *tsst* gene, 23 isolates contained the *sec* gene, 20 isolates contained the *seb* gene, and 2 isolates contained the *SEA* gene (Figure. 2). Also, as presented in Table 3, 11% of the isolates contained three genes, 12% contained two genes, 26% contained one gene, and 51% were devoid of the studied genes.

Staphylococcus spp. were identified based on the *sea*, *seb*, *sec*, and *tsst* genes with accession numbers OQ550021, OP924107, OQ550022, and OP970835. In addition, the results of molecular epidemiology of *S. aureus* species were identified. Phylogenetic tree gene sequences of the sea (A), seb (B), sec (C), and tsst-1 (D) isolates of S. aureus obtained in the present study and those deposited in GenBank with different accession numbers. Accession numbers are shown after the isolate names. The tree was inferred using the neighbor-joining method in MEGA 10. Bootstrap values are shown at each branch point. The numbers above branches reflect bootstrap support of 1000 replicates. All aligned sites containing some degree of deletion or missing data were excluded from the analysis. (Figure 3).

Table 2. Frequency of different toxin-encoding gene

Pattern	Genes						Frequency
	sea	*seb*	*sec*	*see*	*tsst*	*pvl*	
1							51
2	+	+	+				1
3	+						1
4		+					2
5		+	+		+		10
6		+	+				4
7		+			+		3
8			+				4
9			+		+		5
10					+		19

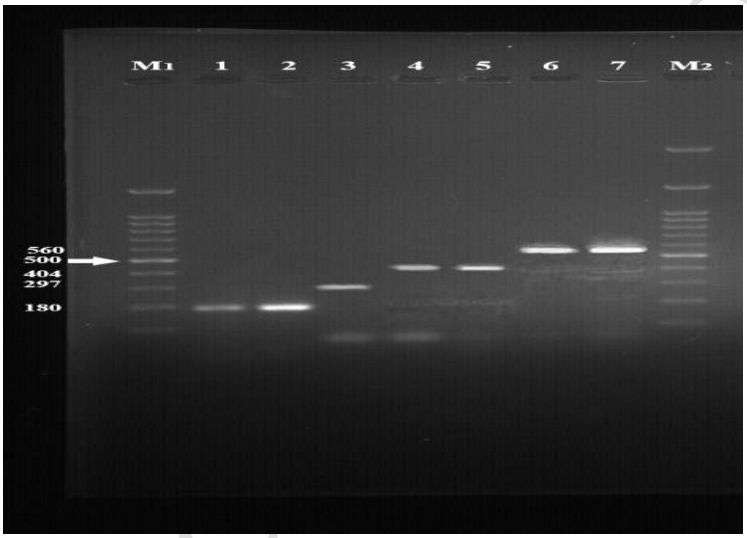

Fig. 2. Gel electrophoresis of *sea*, *seb*, *sec*, *see*, *tsst*-1, and *pvl* genes M1&M2 well: Marker (bp100), wells 1 to 7: *Staphylococcus aureus* positive samples with the *TSST*-1, *SEC*, *SEB*, *SEA* genes, respectively; band sizes are bp 180, bp297, bp404, and bp560, respectively.

Table 3. Results of enterotoxin genes of confirmed *Staphylococcus aureus*

Gene name	**Number of positive isolates**
sea	2 (n=100: 2%: 95%CI: 0.55%-7%)
seb	20 (n=100: 20%: 95%CI: 13.34%-28.88%)
sec	23 (n=100: 23%: 95%CI: 15.84%-32.15%)
see	0 (n=100: 0.0%: 95%CI: 0.0%-3.07%)
tsst	37 (n=100: 37%: 95%CI: 28.18%-46.78%)
pvl	0 (n=100: 0.0%: 95%CI: 0.0%-3.07%)

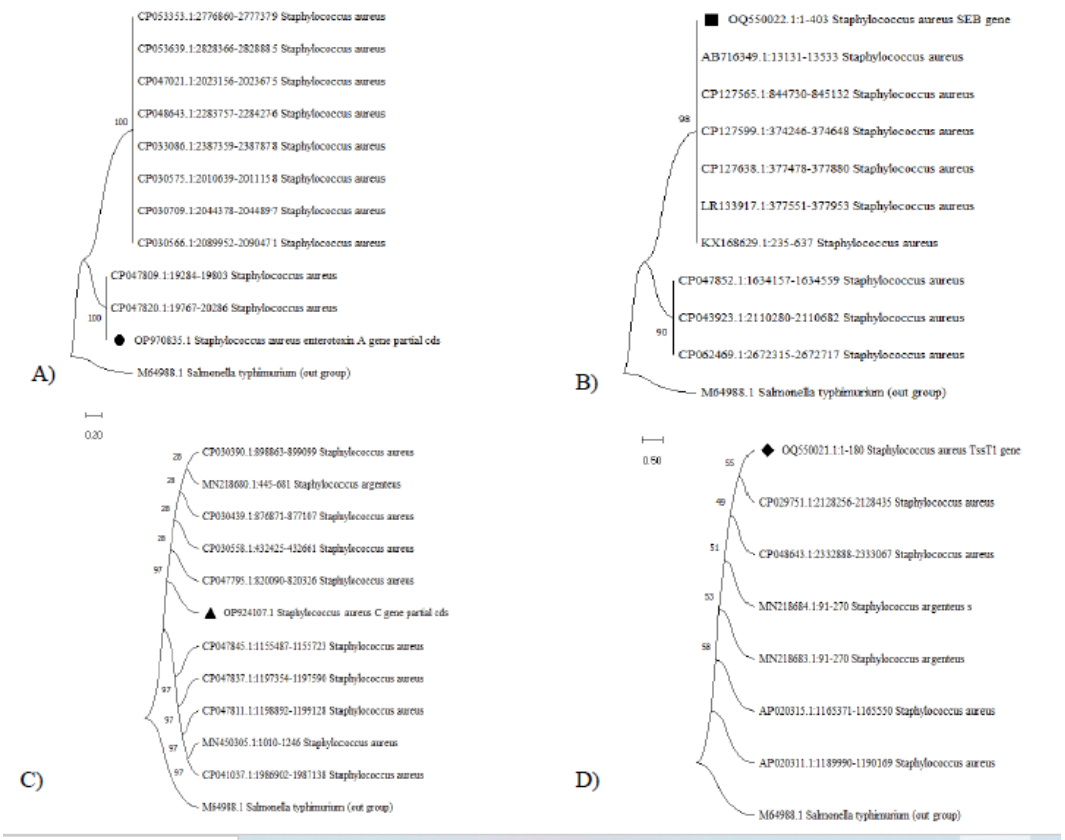

Fig. 3. Phylogenetic tree of the *sea* (A), *seb* (B), *sec* (C), and *tsst*-1 (D) gene sequences of *Staphylococcus aureus*. Accession numbers are shown after the isolate names. tsst-1 gene sequences obtained in this study are shown with bold geometric shapes.

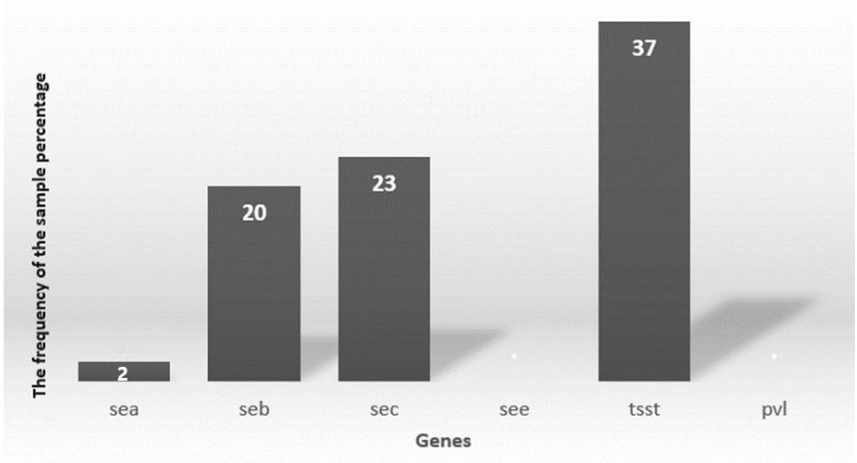

Fig. 4. Number of positive samples in columns

Discussion

Staphylococcus aureus is a human and animal pathogen considered one of the most dangerous pathogens, responsible for a wide range of diseases, from local and systemic infections to toxin-mediated diseases (5, 7). A variety of animal diseases can also be attributed to this bacterium. The main habitat of *S. aureus* is the mucous membranes of the human nose, nasopharynx, and the normal skin flora of warm-blooded animals and humans (8). *Staphylococcus aureus* residing in the nasal cavity of sheep is considered a potential reservoir for staphylococcal infections. It is one of the most common pathogens that cause food poisoning; moreover, it is crucial to recognize that infections attributed to the *sea, seb, sec, see, tsst,* and *pvl* enterotoxin genes are important (9, 10). To date, no study has been conducted to investigate the frequency of toxin-encoding genes on *S. aureus* isolates collected from sheep's noses. As mentioned, staphylococcal enterotoxins have similar biological structural properties, but their expression mechanisms and production levels are different. Staphylococcal enterotoxins A and B are among the most common enterotoxins, so the timely diagnosis and control of strains that produce the genes of these virulence factors are essential. In this study, of the total 100 *S. aureus* isolates, 49 isolates contained at least one enterotoxin gene. The most abundant gene was *tsst* (37%). Then, *sec* 23%, *seb* 20%, and *sea* 2% were identified with lower frequencies. In this study, the most abundant gene was *tsst* (37%). This toxin causes fever, shock, and exfoliative skin rashes. The presence of *S. aureus* in sheep's noses may be dangerous for consumers due to the organism's ability to produce enterotoxin and *TSST* toxic shock syndrome toxin. This toxin can cause severe food poisoning by creating resistance to it. The frequency of enterotoxin genes varies depending on whether the source of the toxin-producing bacterium is animal, human, infection, food, or environment. This is evident in the researchers' report. In a study by Sundararaj et al. (2019), the *TSST* gene of *S. aureus* was identified using the PCR method (11). Hoseini Alfatemi et al.'s findings in 2014 showed that 37% of the isolated staphylococci contained the *TSST* gene (11). This is in agreement with the percentage of abundance obtained from sheep's nose isolates in the present study. In a study by Fathali et al. (2015), 14 isolates (35%) of *S. aureus* had the *SEC* gene, and 80 isolates (17.5%) had the *TSST* gene (12). In Germany in 2019, Becker and colleagues identified 40 *S. aureus* isolates (18.6%) with the *tsst* gene and 19 isolates (8.6%) with the *sec* gene from 59 isolates (13).

In a study by Manfredi and colleagues in 2010, 24 isolates (23.3%) had the *sea* gene, and nine isolates (9.5%) had the *sec* gene (14). In a study by Soltan Dallal and colleagues in 2012 in Slovakia, 8.5% had the *sea* gene, 10% had the *seb* gene, 45.5% had the *sec* gene, and 43% had the *tsst* gene from isolates of *S. aureus* isolated from animal sources (15). In a study conducted by Kwon et al. in 2004, it was found that 30% of the 14.19% of *S. aureus* isolates collected from sheep in South Korea had the *SEA* gene (16). In a study conducted by Omoe et al. in 2002, it was reported that 66 of the 146 *S. aureus* isolates collected from animals had at least one enterotoxin gene, of which 76% had the *sea* gene and 16.9% had the *seb* gene (17). In a study conducted by Chiang et al. in 2006, 74.1% of *S. aureus* isolates collected from animals had genes encoding enterotoxin A and C. In the same study, 28.6% of the *S. aureus* isolates collected from animals had the *sea* gene and 8.2% had the *sec* gene (18). In 2009, Peck et al. found that 34.3% of the 70 clinical samples of *Staphylococcus aureus* examined in Korea South were positive for the *sec* gene (19). Aslanimehr et al. found that 27% of the 65 *S. aureus* isolates they studied in Iran were positive for the *tsst* gene and 3% for the *sec* gene (20). Baz et al. found that none of the 96 *S. aureus* isolates they studied in clinical samples were positive for the *see* gene, similar to our study (21). Becker et al. found that 18.6% of the 59 *Staphylococcus aureus* isolates they studied in Germany were positive for the *tsst* gene, and 8.6% were positive for the *sec* gene14 Havaei et al. found that none of the 149 *S. aureus* isolates they to

studied in Iran were positive for the *pvl* gene in 2010 (22). Shohayeb et al. found that 18% of the 100 *S. aureus* isolates they studied in Egypt were positive for the *pvl* gene in 2023 (23). A study conducted in 2007 by Yu et al., reported that 25 (12.8%) of 195 *S. aureus* isolates were positive for Panton-Valentine leukocidin (pvl) genes in a teaching hospital in Wenzhou, China. Nineteen (11.9%) of 160 hospital-acquired isolates, and six (17.1%) of 35 community-acquired isolates, harboured lukS/F-PV (24). One of the notable findings of the present study is that the results of this study are consistent with the results of numerous studies conducted worldwide. Still, they differ from the results of some other studies that have been done. The difference in the prevalence rate can be related to the following factors: the difference in the population under study, the different sampling and culture methods used, the type of samples, the difference in the number of samples under study, the difference in the sampling location, and the difference in the origin of the *S. aureus* samples. In other studies, it has been shown that the prevalence of *S. aureus* strains that produce enterotoxin varies depending on the human, animal, or environmental origin.

Conclusion

In the present study, a multiplex PCR technique was used to identify genes responsible for producing enterotoxins. This is a specific, sensitive, rapid, and inexpensive method. This technique can simultaneously detect several enterotoxin-producing genes. Considering that *S. aureus* strains that produce enterotoxin, especially the TSST gene, play a role in the severity of the disease, other genes mentioned above are also involved. On the one hand, the enterotoxins of this bacterium are heat-resistant and do not disappear during the heating and cooking stages. Therefore, due to the high prevalence of enterotoxin genes isolated from *S. aureus* in the noses of apparently healthy sheep, PCR testing can be useful for identifying strains with the genes for enterotoxins A, C, B, E, TSST, and PVL in sheep. In general, the presence of enterotoxigenic strains of *S. aureus* can pose a potential health hazard.

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Ethical approval

Our research was conducted in accordance with the guidelines and standards of the Animal Research Ethics Committee of the Zabol University**.** However, the nose swap sample was from a live sheep and there was no need to receive the code of ethics.

Conflict of interest statement

There is no conflict of interest.

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