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# Investigation of Enterotoxin-Producing Genes (*sea*, *seb*, *sec*, and *sed*) in *Staphylococcus aureus* Isolated from Raw Traditionally and Pasteurized Milk Supplied in Tehran, Iran

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## HIGHLIGHTS

- Totally, 32 (21.3%) samples of raw traditional milk and one sample (6.7%) of pasteurized milk samples were infected with *Staphylococcus aureus*.
- The frequency of presence of *sea*, *seb*, *sec*, and *sed* genes was: 9 (28.12%), 14 (43.75%), 6 (18.75%), 2 (6.25%), respectively.
- Totally 38 (33.25%) of raw traditional milk samples and 5 (33.33%) of pasteurized milk had antibiotic residue.
- Having a properly monitoring on the using of antibiotic in livestock farms is seen necessary.

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SE=Staphylococcal Enterotoxin

## ABSTRACT

**Background:** The issue of milk quality appears to be vital due to its nutritional value and since raw milk can be regarded as an appropriate environment for the growth of several pathogens by producing an enterotoxin. The aim of present study is to investigate enterotoxin-producing genes (*sea*, *seb*, *sec*, and *sed*) from *Staphylococcus aureus* isolated from raw traditional and pasteurized milk in Tehran, Iran.

**Methods**: One hundred and fifty samples of raw traditional milk supplied in five districts of Tehran were collected and simultaneously15 pasteurized milk samples from various brands were prepared and examined phenotypically and bio-chemically for the existence of *S. aureus*. The presence of *sea*, *seb*, *sec*, *sed* genes was assessed by Polymerase Chain Reaction and ultimately the antibiotic residue was measured with a commercial kit.

**Results**: In this study, 32 (21.3%) samples of raw traditional milk and one sample (6.7%) of pasteurized milk samples were infected with *S. aureus.* The frequency of presence of *sea*, *seb*, *sec*, and *sed* genes regarded to be: 9 (28.12%), 14 (43.75%), 6 (18.75%), 2 (6.25%), respectively however *sec* gene failed to identify. Basically 38 (33.25%) of raw traditional milk samples as well as 5 (33.33%) of pasteurized milk included antibiotic residue.

**Conclusion:** The high prevalence of *S. aureus* comprising enterotoxin genes in raw traditional milk is considered as a severe warning to the community and highlights the need for a high quality product.



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## Introduction

Zoonosis is known as an infectious disease that can be transmitted between vertebrates and humans. The term is often applied specifically to refer to diseases that originate in other animal species and are transmitted to humans (Bardosh, 2016). According to the World Organization for Animal Health, the fight against zoonoses diseases innitiates with the elimination of the pathogen in the animal source. Approaches to control such diseases should be focused on animal products for attaining this goal (Verma et al., 2014). A systematic study indicated that out of 1,415 known pathogens for humans, 61% (868) are diseases that can be transmitted between humans and animals. It is also crucial to cosider that more than 70% of emerging diseases are zoonosis in which animals act as reservoir hosts. One of the most significant organisms consisted in the development of zoonotic diseases are bacteria (Sabour et al., 2022). Food-borne diseases are considered as a major public health problem that affects millions of people worldwide annually at a cost of billions of dollars, and some of whom die or are hospitalized (Peles et al., 2007). *Staphylococcus aureus* is the second or the third most fundamental cause of these diseases. Food products are considered as important sources that can transmit this bacterium to humans and can lead to disease in both humans and animals. This bacterium can be isolated from a variety of foods consisting of dairy products especially milk, meat products, vegetables, salads, cooked, and salty foods especially foods requiring long manipulations due to the ease of growth in different conditions (Kapoor et al., 2023). *S. aureus* exhibits several pathogenic factors that determine the pathogenicity and colonization of the bacterium. Staphylococcal Enterotoxins (SEs) proved to be a family of of heat stable enterotoxins. Heat-resistant enterotoxins are the leading cause of gastritis and intestinal inflammation which are engendered by eating contaminated food. Furthermore, SE appears to be superantigens that stimulate nonspecific T cell proliferation. SEs are considered phylogenetic by similar structures and activities as well (Balaban and Rasooly, 2000). Among SEs, *sea* and *seb* are turned out to be more specific and significant due to their better ability to bind to Major Histocompatibility Complex (MHC) class II molecules and greater stimulation of T cells. (Pinchuk et al., 2010). Although the most common SEs are *sea* and *seb* enterotoxins (Ler et al., 2006), studies have identified that *sed* is one of the distinguished and common SE associated with food poisoning (Khoothiam et al., 2023). The other SEs are *see* and *sef* that related in several cases of food poisoning and toxic shock syndrome, respectively (Oliveira et al., 2022). For instance, a study of methicillin and enterotoxin resistance genes and the pattern of antibiotic resistance of *S. aureus* in raw milk revealed that about 45.7% of *S. aureus* contained the genes *sea*, *seb*, *sec*, *sed*, *see* (Riva et al., 2015). The use of dairy products, especially milk, as the cheapest protein in the daily basket of the household, and despite the tendency of people in consuming traditional dairy products, especially milk and yogurt, provides the basis of food poisoning. This study made an attempt to isolate and evaluate enterotoxin-producing genes (*sea*, *seb*, *sec*, *sed*) of *S. aureus* which isolated from traditionally supplied raw and pasteurized milk. Furthermore, the antibiotic residue in milk was meseaured.

## Materials and methods

### *Sampling*

In a cross-sectional descriptive study (during August-December in 2021), 30 samples of raw traditional milk from each of the five districts of Tehran (North, South, East, West, and Center) were collected in a total of 150 samples under hygienic conditions. Also, 15 samples of pasteurized milk with various brands were prepared as controls.

### *S. aureus detection*

One ml of milk was added to 9 ml of Giolitti Cantoni broth (Merck, Germany). After the mixing of the contents with liquid paraffin, it was covered and incubated for 24 h at 37 °C. One loop of the contents was transferred to Baird-Parker agar medium (Merck, Germany) and cultured streakingly. Glossy black, convex colonies with oil sediment margin were regarded as suspected colonies of *S. aureus* and then catalase, coagulase, mannitol fermentation; After Gram staining, DNase tests were performed for phenotypic confirmation (Soltan-Dallal et al., 2010).

### *Antibiotic residues determination*

To determine the antibiotic residue in milk qualitatively, Hansen's kit (Denmark) was applied and based on kit protocol, placed in a specific microbiological test incubator. The milk was poured into a vial containing the lyophilized antibiotic and placed in an incubator at 64 °C for 3 h. If antibiotics are present in the milk, the contents of the kit will shift from purple to yellow.

### *Molecular identification*

At first, genomic DNA was extracted by boiling method (Rahimkhani and Rajabi, 2022). Subsequently, amplification was carried out using the specific primer pairs (Table 1) and with the temperature conditions displayed in Table 2 using the PeQlab thermo cycler instrument (peQSTAR, China). The amplification analysis



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was performed by 1% electrophoresis with 5 V/cm. Finally, visualization was performed by the gel documentation system (Cleaver, UK).

## Results

The isolation and identification results demonstrated that out of 150 raw milk samples, 70 (46.7%) were infected with Staphylococci. Thirty two (21.3%) samples were positive for *S. aureus* and 38 (25.3%) samples were positive for coagulase-negative Staphylococci. Out of 15 tested samples of pasteurized milk one sample (6.7%) was infected with *S. aureus* (Table 3).

*Antibiotic residue*

Out of 165 milk samples tested for antibiotic residue (150 samples of raw milk and 15 samples of pasteurized milk), 43 (26.06%) milk samples, consisting of 38 (33.25%) samples of raw milk and five (33.33%) samples of pasteurized milk contained antibiotic residue.

*Molecular identification*

Out of 32 isolated *S. aureus*, 23 (71.87%) comprised of at least one gene from the four genes studied. The *sea* gene was proved to be as the most predominant enterotoxin gene (14 (43.75%)). The frequency of the other genes was as follow: *seb* 6 (18.75%), and *sed* 2 (6.2%). The *sec* gene was not detected. One strain (3.12%) contained *sea* and *sed* genes simultaneously. The results of agarose gel electrophoresis for isolates containing these genes are demonstrated in the Figure 1.

**Table 1**: The primers used in this study

| Target gene | Primer sequence (5´-3´) | PCR product (bp) | Reference |
|---|---|---|---|
| ***sea*** | F: AAA GTG CCG ATC AAT TTA TGC CTA<br>R: GTA ATT AAC CGA AGG TTC TGT AGA | 219 | Bendahou et al. (2009) |
| ***seb*** | F: TCG CAT CAA ACT GAC AAA CGA<br>R: CAC TTT TTC TTT GTC GTA AGA TAA | 410 | |
| ***sec*** | F: AAC ATT AGT GAT AAA AAA GTG AAA<br>R: TTG TAA GTT CCC ATT ATC AAA GTG | 234 | |
| ***sed*** | F: GCA GAT AAA AAT CCA ATA ATA GGA-<br>R: TAC TAA AGA AAC TTC TTT TTG TAC - | 331 | |

PCR=Polymerase Chain Reaction

**Table 2**: Polymerase Chain Reaction (PCR) temperature conditions

| No | Reaction stage | Temperature (°C) | Time | Repeat number |
|---|---|---|---|---|
| 1 | Predenaturation | 95 | 5 min | 1 |
| 2 | Denaturation | 95 | 30 sec | 35 |
| | Annealing | *sea*, *seb*, *sec*: 50<br>*sed*: 52 | 1 min | |
| | Extension | 72 | 1 min | |
| 3 | Final extension | 72 | 5 min | 1 |

**Table 3**: Distribution of *Staphylococcus aureus* in milk samples isolated from five areas of Tehran

| | North of Tehran | South of Tehran | East of Tehran | West of Tehran | Central part of Tehran |
|---|---|---|---|---|---|
| Percentage of *Staphylococcus aureus* | 4 (12.5%) | 10 (31.2%) | 5 (15.6%) | 7 (21.9%) | 6 (18.6%) |

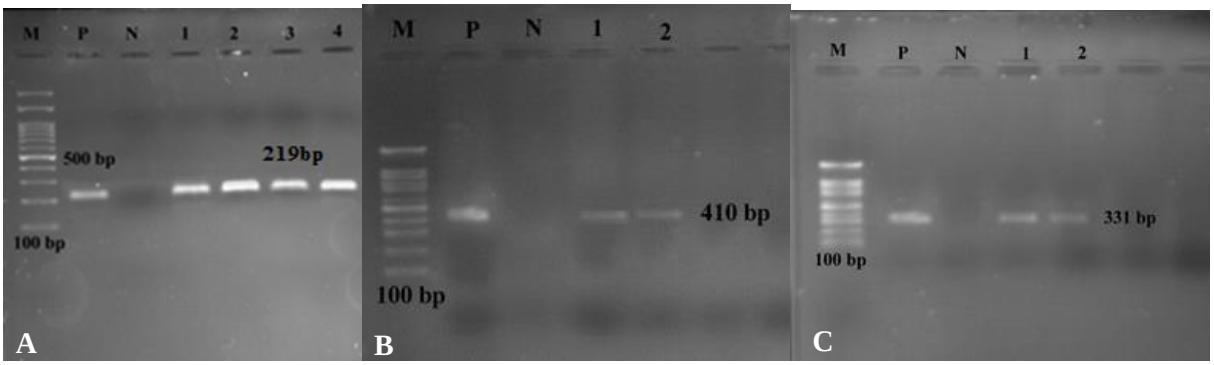


**Figure 1:** Polymerase Chain Reaction (PCR) amplification of enterotoxin genes and toxic shock syndrome toxin-1 gene**. A**: Lanes 1-4: isolates of *Staphylococcus aureus* containing *sea* gene; **B**: Lanes 1-2: isolates of *S. aureus* containing *seb* gene; **C**: Lanes 1-2: isolates of *S. aureus* containing *sed* gene. M: 100 bp DNA ladder; N: Negative control; P: Positive control





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## Discussion

Raw milk is considered as a rich product nutritionally and an appropriate environment for the growth of microorganisms. Consequently, it can be contaminated with a variety of Gram-negative and Gram-positive bacteria. A great number of bacteria isolated from raw milk are regarded as opportunistic pathogens. Food-borne illness turns to be one of the prominant health challenges worldwide (Tirado et al., 2010). According to the Centers for Disease Control and Prevention (CDC), food-borne illnesses in the United States result in the hospitalization of a great number of infected individuals, and death of some persons (Bertolatti and Theobald, 2011). Because of pathogenic factors, *S. aureus* is proved to be as one of the most key pathogens including enterotoxin genes. In this study, out of 150 samples of raw milk, 70 samples (46.7%) were contaminated with *Staphylococcus* species, of which 32 (21.3%) samples were positive for *S. aureus* and 38 (25.3%) samples were positive with coagulase-negative staphylococci. Moreover, out of 15 samples of pasteurized milk tested, one sample (6.7%) was contaminated with *S. aureus*. Milks prepared from the South of Tehran had the highest number of *S. aureus* 10 (31.2%).

Pyrogenic Toxin Superantigens (PTSAgs) are significant virulence factors of this bacterium. Currently, 23 enterotoxins have been identified that are serologically distinct. The most essential enterotoxins include *sea*, *seb*, *sec*, and *see* (Mashouf et al., 2015). According to the findings of the current study, 23 isolates of *S. aureus* have included *sea*, *seb*, and *sed* genes. However, there was no result for *sec*.

Based on the study by Haghi et al. (2019) in Iran, more than 80% of the strains produced enterotoxin, and gene *sea* was the most abundant (88.2%), as well as *seb* and *sed* had the same frequency (52%). About 70% of the isolated carried two genes at the same time. In addition, 76.5% of the isolates exhibited two or more genes simultaneously (Haghi et al., 2019). Moreover, Morandi et al. (2007), identified that 67% of *S. aureus* isolates had enterotoxin genes, and these isolates often had *sea*, *sed*, and *sej* enterotoxins. Many researchers have proved in their studies that *sea* is the most prevalence enterotoxin gene in isolated staphylococci from raw milk (Haghi et al., 2019; Moradi Farsani et al., 2018; Morandi et al., 2007). Although, *sea* has been presented to be the most common cause of food poisoning in some countries (Hwang et al., 2007; Riva et al., 2015). In the study conducted by Zhao et al. (2021), among 95 *S. aureus* isolates from raw milk, *sed* (13.2%) and *sec* (8.3%) were recognized as the most frequent enterotoxin genes. In addition, Kou et al. (2021) presented that *see* is the most prevalent enterotoxin gene in the *S. aureus* isolated from retail raw milk. Overally, according to published results, *sea* is the most common enterotoxin in enterotoxigenic strains of *S. aureus* and plays the most prominant role in the incidence of staphylococcal food poisoning (Soltan Dallal et al., 2018).

The ability to produce various enterotoxins can be related to the ecological origin of the bacterium, the nature of the isolates, the growth medium and the nutrients available to the bacteria, the type of food and even the samples. In cases of poisoning caused by contaminated dairy products, *sea* has the highest frequency while in other food stuffs such as meat samples, *sec* has the highest frequency.

## Conclusion

In the current study, the frequency of isolated enterotoxigenic *S. aureus* in traditional raw milk is considerable. On the other hand, the presence of antibiotic residue in raw traditional milk manifested that the risk of antibiotic presence in milk can be a warning of antibiotic resistance for humans due to consumption. Having a proper monitoring on the use of antibiotic in livestock farms is considered as a necessary fact. Observing the principle of pasteurization in the use of raw traditional milk is necessary since the frequency of enterotoxigenic *S. aureus* is regarded significant.

## Author contribution

Conceptualization: M.M.S.-D.; methodology: Z.R. and M.M.S.-D.; investigation: Z.R., A.M.S., M.Z., S.S.Y., S.S.Y., and P.T.B.; writing the original draft: S.Z.M. and Z.R.; writing the review and editing: M.M.S.-D. and S.Z.M.; analyse data acquisition: Z.R. and M.M.S.-D. The final manuscript was read and approved by all authors.

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## Conflict of interest

The authors declare that there is no conflict of interest.

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