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The Frequency of *Staphylococcus aureus* Classical Enterotoxin Genes in Raw Milk Samples in Zanjan, Iran

Fakhri Haghi [a] | **Shahrzad Daneshamooz** [a] | **Angineh Parsadanians** [a] | **Habib Zeighami** [ab*]

[a] *Department of Social Determinants of Health Research Center, Zanjan University of Medical Sciences, Zanjan, Iran.*
[b] *Department of Microbiology, Zanjan University of Medical Sciences, Zanjan, Iran.*

***Corresponding author:** Habib Zeighami
Department of Microbiology, Zanjan University of Medical Sciences, Zanjan, Iran. Postal code: 4513956111.
E-mail address: zeighami@zums.ac.ir

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ABSTRACT

Background: *Staphylococcus aureus* is one of the major causes of food poisoning. Since milk is a nutritious source of proteins and vitamins, it could provide the optimal conditions for the growth of several bacterial pathogens, such as *S. aureus*. The present study aimed to assess the frequency of *Staphylococcus aureus* classical enterotoxin genes in raw milk samples in Zanjan, Iran.

Methods: In total, 82 bovine, unpasteurized milk samples were collected from the dairy farms in various rural areas in Zanjan, Iran. The isolation and identification of *S. aureus* were performed using the Baird-Parker agar, routine biochemical tests, and polymerase chain reaction (PCR) targeting the *S. aureus*-specific *femA* gene. In addition, staphylococcal enterotoxin genes (e.g., *sea*, *seb*, *sec*, *sed*, and *see*) were assessed using PCR.

Results: Following the appearance of yellow colonies with yellow zones on Mannitol salt agar, 21 *S. aureus* isolates (25.6%) were detected. In total, 80.9% of the isolates were positive for the presence of SE genes, and the most frequent SE gene was *sea* (88.2%), followed by *see* (58.8%), and *seb* (52.9%). Furthermore, 76.5% of the isolates had two or more SE genes simultaneously.

Conclusion: According to the results, the presence of enterotoxigenic *S. aureus* in the studied raw milk samples confirmed the possible risk posed on the public health. Therefore, it is recommended that the quality of dairy product quality programs be optimized in order to intensify the sanitary inspection of these products.

1. Introduction

Staphylococcus aureus is a gram-positive, non-spore-forming, immotile coccus belonging to the family Micrococcaceae, which is considered to be a leading cause of bacterial food poisoning outbreaks [1-3]. Staphylococcal food poisoning (SFP) is caused by the consumption of contaminated foods containing enterotoxins. Approximately 20-30% of human populations are consistent carriers of this bacterium, while 60% are the transient carriers of *S. aureus*. Therefore, the insufficient pasteurization and decontamination of food products or their contamination during preparation, processing, and distribution by the carriers of *S. aureus* are the common risk factors for the outbreaks of staphylococcal food poisoning [3-6].

Some of the food products that are often involved in the transmission of staphylococcal poisoning include meat and its products, poultry and egg products, milk and dairy products, salads, bakery products (especially cream-filled pastries and cakes), and sandwich fillings [7-11]. The most common symptoms of staphylococcal food poisoning are nausea and vomiting, diarrhea, and abdominal cramps, which occur within 2-6 hours after the consumption of enterotoxin-containing foods. Occasionally, SFP may be more severe or even fatal, especially in infants, the elderly or immunocompromised patients [11, 12].

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Staphylococcal enterotoxins (e.g., SEs, SEA-SEE, SEG-SEI, and SER-SET) and staphylococcal enterotoxin-like toxins (e.g., SEls) have been reported to be involved in food poisoning; such examples are SElK-SElQ and SElU-SElX. SEs and SEls are single-chain proteins with the size range of 22-29 KDa and encoded by various genetic elements, such as plasmids, bacteriophages, pathogenicity islands, vSA genomic islands, and staphylococcal cassette chromosome [12-15]. Extremely low amounts of SE (approximately 20 ng-1 μg) in food products are required to develop food poisoning [7, 16, 17]. Due to the stability of SEs at high temperatures and low pH, these toxins are not completely destroyed by the mild cooking or digestion of food in the stomach [18], thereby posing significant risks to the health of the consumers after using unpasteurized milk. During 2011-2014, 4,211 foodborne disease outbreaks were reported in China, in which *S. aureus* was recognized as one of the most prominent cause of the disease in 3,269 of the cases [7, 19].

The consumption of homemade dairy products, such as raw milk, is associated with severe public health hazards [12]. Despite the governmental surveillance of milk pasteurization and sanitation in dairy processing plants for several years, the direct sale of unpasteurized milk and dairy products is rather common in many regions in Iran, such as Zanjan province. Therefore, it is essential to obtained adequate data on the microbial risk factors associated with the production of raw milk. Risk assessment and microbial monitoring also play a pivotal role in the quality assurance of milk and its products [12]. To the best of our knowledge, data is scarce regarding the frequency of *S. aureus* and enterotoxin genes in raw milk samples in Iran .

The present study aimed to investigate the frequency of *S. aureus* and classical enterotoxin genes in the bacterial strains isolated from the raw milk samples collected from the dairy herds in Zanjan, Iran. This is the first report on the frequency of enterotoxigenic S. aureus in raw milk samples in Zanjan, Iran.

2. Materials and Methods

2.1. Collection of Milk Samples

During March-June 2017, 82 bovine, unpasteurized milk samples (one sample per animal) were collected from the traditional dairy farms in various rural areas in Zanjan, Iran. The animals from which the milk samples were obtained for the study were clinically healthy, and the milk samples showed physicochemical consistency in terms of color, pH, and density. The milk samples were collected in 50-milliliter sterile centrifuge tubes (SPL Life Sciences, Gyeonggi-do, Korea) and immediately transferred to the laboratory of food microbiology in a chilled box for further analysis within one hour.

2.2. Reference Strains

The reference strains used as the positive controls in the present study included *S. aureus* ATCC 13565 (SEA), *S. aureus* ATCC 14458 (SEB), *S. aureus* ATCC 19095 (SEC), *S. aureus* ATCC 23235 (SED), and *S. aureus* ATCC 27664 (SEE).

2.3. Isolation and Identification of S. aureus

The milk samples were centrifuged at 5,000 rpm for five minutes, and bacterial pellets were streaked onto the Baird-Parker agar (Merck, Darmstadt, Germany) and incubated in aerobic conditions at the temperature of 37 °C for 24 hours. Afterwards, the grey-black colonies were sub-cultured onto the Mannitol salt agar (Merck, Darmstadt, Germany) and incubated at the temperature of 37°C for 24 hours. In addition, the suspected *S. aureus* colonies were identified using routine biochemical tests, including gram staining, catalase, coagulase, oxidase, lipase, DNase tests, and PCR targeting the S. aureus-specific femA gene (specific to the S. aureus species. The primer sequence in this experiment is presented in Table 1 [20].

2.4. Genomic DNA Extraction

A colony of *S. aureus* was collected from the nutrient agar and inoculated into two milliliters of Luria Bertani broth (Merck, Darmstadt, Germany) until achieving the exponential phase with 2 McFarland turbidity (6×10 8 CFU/ml) and shaking at 120 rpm and the temperature of 37 °C. The extraction of genomic DNA was performed in accordance with the protocol of GeneAll Exgene Genomic DNA Micro (GeneAll Biotechnology, Seoul, Korea).

2.5. Detection of sea and see Enterotoxins Using PCR

Staphylococcal enterotoxin genes (*sea, seb, sec, sed,* and *see*) were assessed using the primers shown in Table 1. Simplex PCR was performed using DreamTaq PCR Master Mix (Ampliqon, Denmark), which contained Taq polymerase, dNTPs, MgCl2, and an appropriate buffer. In addition, each PCR tube contained 25 microliters of the reaction mixture, which was composed of 12.5 microliters of the master mix, one microliter of each forward and reverse primer solution (final concentration: 200 nM), one microliter of DNA (concentration: 100 ng/μl), and nuclease-free water to achieve the final volume.

At this stage, PCR was performed using the Gene Atlas 322 system (ASTEC) with the same cycling conditions for the sea-see genes. Amplification involved initial denaturation at the temperature of 94 °C for five minutes, followed by 30 cycles of denaturation (94°C, 1.5 minutes), annealing (55 °C, 1.5 minutes), extension (72 °C, 1.5 minutes), and final extension (72 °C, 8 minutes). Following that, the amplified DNA was separated via submarine gel electrophoresis on 1.5% agarose, stained with ethidium bromide, and visualized using a UV transilluminator.

Table 1: Primers used in this study

Target	Primer sequence (5′→3′)	Amplicon size (bp)	Ref.
f*emA*	AAAAAAGCACATAACAAGCG AAAAAAGCACATAACAAGCG	132	[20]
sea	CCTTTGGAAACGGTTAAAACG TCTGAACCTTCCCATCAAAAAC	127	[30]
seb	TCGCATCAAACTGACAAACG GCAGGTACTCTATAAGTGCC	477	[30]
sec	CTCAAGAACTAGACATAAAAGCTAGG TCAAAATCGGATTAACATTATCC	271	[30]
sed	CTAGTTTGGTAATATCTCCTTTAAACG TTAATGCTATATCTTATAGGGTAAACATC	319	[30]
see	CAGTACCTATAGATAAAGTTAAAACAAGC TAACTTACCGTGGACCCTTC	178	[30]

3. Results and Discussion

Food safety is considered to be a major global health concern with international trade and public health implications. S. aureus is the most common cause of food poisoning and a major public health concern in developing countries [21, 22]. Staphylococcal food poisoning due to the consumption of raw milk has been reported in various studies [20, 22-24]. In the current research, 82 bovine raw milk samples were assessed in terms of the presence of *S. aureus.* Following the appearance of yellow colonies with yellow zones on the Mannitol salt agar, 21 *S. aureus* isolates (25.6 %) were detected. Furthermore, the biochemical tests and molecular analysis of the femA gene in the coagulase-positive staphylococci confirmed the presence of these isolates. Similar to our findings, Rahbar Saadat et al. (2014) detected *S. aureus* in 27% of milk and cheese samples [25].

To the best of our knowledge, there is inadequate data regarding the frequency of *S. aureus* and enterotoxin genes in the raw milk samples in Iran. This is first report on the frequency of enterotoxigenic *S. aureus* in the raw milk samples in Zanjan province. Most of the studies in this regard have been focused on enterotoxigenic *S. aureus* in specific geographical areas, while a nationwide surveillance system has not been established yet.

According to the study by Hassani et al. (2014) in Iran, 43% of dairy samples were contaminated with *S. aureus*, with 22% of the bacterium detected in the milk samples and 18% detected in the cheese samples [23]. Inconsistent with the results of the present study, higher frequency of *S. aureus* was reported in domestic dairy products in a previous study conducted in Iran. According to the mentioned study, 32% of the dairy products were contaminated with *S. aureus* (cream: 18%, cheese: 10%, milk: 4%) [24]. Although the health regulations in Iran have not established any limits for the permitted level of *S. aureus* in milk, it is known that the enterotoxins produced by these isolates could reach sufficient levels to cause food poisoning symptoms of the *S. aureus* concentrations exceeded 105 CFU/ml [26]. The frequency of raw milk contamination with *S. aureus* has been reported to be lower (7.3%) in São Paulo state, Brazil (2010). According to the results of the mentioned study, *S. aureus* strains were detected in 14 cow milk samples (6.7%) and four bulk tank milk samples (10.8%) [27]. The variation in the frequency of *S. aureus* in dairy products may be due to the differences in the geographical region, number of samples, season of sampling, post-harvest practices, the hygienic standards that are applied during the handling, transport, and storage of dairy products, and methods used for the isolation and identification of S. aureus [11].

According to the findings of the current research, 80.9% of the isolates (n = 17/21) were positive for the presence of at least one or more SE genes. The most prevalent SE gene among the isolates detected in the raw milk samples was *sea* (n = 15/17, 88.2%), followed by *see* (n = 10/17, 58.8%) and *seb* (n = 9/17, 52.9%). Moreover, the frequency of the *sec* and *sed* genes was estimated at 23.5% and 17.6%, respectively. Consistent with the results of the present study, SEA has been reported to be the most common cause of food poisoning in Korea and Japan [28].

The previous studies in this regard have also indicated that *sea* and *sed* are the most common enterotoxin genes in the staphylococci isolated from food products [29]. However, the frequency of *sed* (17.6%) was observed to be lower in the isolates of the raw milk samples in the current research. In contrast, *seb* has been observed to be a prevalent gene in patients with food poisoning in Taiwan and Japan, while *sec* has been reported to be a major SE gene in the isolates found in the bulk milk samples in Switzerland and Korea [28].

In current research, multiple SE genes were present with various combinations in the studied isolates (Table 2). Among 17 *S. aureus* isolates carrying enterotoxin genes, 13 cases (76.5%) contained two or more SE genes simultaneously. In this regard, the most frequent combination of SE genes was *sea+see* (23.5%), followed by *sea+seb+see* (17.6%). Furthermore, one isolate (5.8%) carried the sea, seb, sec, and see genes simultaneously.

One of the limitations of the present study was the small sample size. Furthermore, due to the lack of funding, it was not possible to investigate the frequency of *S. aureus* in other dairy products, such as cheese, cream, butter, yoghurt, and Kashk.

Table 2: SE combinations among 17 *S. aureus* isolates carrying enterotoxin genes.

SE combinations	No. (%) of isolates
sea+sed	1 (5.8)
sea+seb	2 (11.7)
sea+see	4 (23.5)
sec+sed	1 (5.8)
sea+seb+see	3 (17.6)
sea+sec+see	1 (5.8)
sea+seb+sec+see	1 (5.8)

4. Conclusion

Raw milk is widely available in the marketplace in Zanjan, Iran. Therefore, it is essential to collect adequate data regarding the microbial risk factors and health hazards associated with the production of raw milk. Critical control point management programs should be developed for milk production farms individually based on risk assessment and total quality management. Moreover, critical control point principles (e.g., pasteurization and sanitary treatment of milk) are essential to obtaining safe and healthy milk for processing and consumption.

Authors' Contributions

Project development, Manuscript writing, Data analysis: F.H., Data collection, Project development: Sh.D., and A.P., Project development, Data management, Data analysis, Manuscript writing: H.Z., All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

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References

1. Imani Fooladi A, Riazipour M, Sattari M. Molecular and Serological Detection of Enterotoxigenic *Staphylococcus aureus* from Traditionally Dairy Products. *J Shahrekord Univ Med Sci*. 2010; 11(4): 19-26.
2. Jahanshahi A, Zeighami H, Haghi F. Molecular Characterization of Methicillin and Vancomycin Resistant *Staphylococcus aureus* Strains Isolated from Hospitalized Patients. *Microb Drug Resist*. 2018; 24 (10): 1529-36.
3. Kluytmans JA, Wertheim HF: Nasal Carriage of *Staphylococcus aureus* and Prevention of Nosocomial Infections. *Infection*. 2005; 33(1): 3-8.
4. Udo EE, Al Mufti S, Albert MJ. The Prevalence of Antimicrobial Resistance and Carriage of Virulence genes in *Staphylococcus aureus* Isolated from Food Handlers in Kuwait City Restaurants. *BMC Res Notes*. 2009; 2: 108-14.
5. Fetsch A, Contzen M, Hartelt K, Kleiser A, Maassen S, Rau J, et al. *Staphylococcus aureus* Food-Poisoning Outbreak Associated with the Consumption of Ice-Cream. *Int J Food Microbiol*. 2014; 187: 1-6.
6. Tiemersma EW, Bronzwaer SL, Lyytikainen O, Degener JE, Schrijnemakers P, Bruinsma N, et al. Methicillin-Resistant *Staphylococcus aureus* in Europe, 1999-2002. *Emerg Infect Dis*. 2004; 10(9): 1627-34.
7. Hennekinne JA, De Buyser ML, Dragacci S. *Staphylococcus aureus* and Its Food Poisoning Toxins: Characterization and Outbreak Investigation. *FEMS Microbiol Rev*. 2012; 36(4): 815-36.
8. Letertre C, Perelle S, Dilasser F, Fach P. Identification of a New Putative Enterotoxin SEU Encoded by the Egc Cluster of *Staphylococcus aureus*. *J Appl Microbiol*. 2003; 95(1): 38-43.
9. Akineden O, Hassan AA, Schneider E, Usleber E. Enterotoxigenic Properties of *Staphylococcus aureus* Isolated from Goats' Milk Cheese. *Int J Food Microbiol*. 2008; 124(2): 211-6.
10. Tkáčiková Ľ, Tesfaye A, Mikula I. Detection of the Genes for *Staphylococcus aureus* Enterotoxins by PCR. *Acta Veterinaria Brno*. 2003; 72(4): 627-30.
11. Asgarpoor D, Haghi F, Zeighami H. Frequency of Enterotoxin Producing *Staphylococcus aureus* and Toxin Genes in Raw and Cooked Meat Samples. *Infect Epidemiol Microbiol*. 2018; 4(2): 53-8.
12. Haghi F, Zeighami H, Naderi G, Samei A, Roudashti S, Bahari S, et al. Detection of Major Food-Borne Pathogens in Raw Milk Samples from Dairy Bovine and Ovine Herds in Iran. *Small Ruminant Res*. 2015; 131: 136-40.
13. Podkowik M, Park JY, Seo KS, Bystron J, Bania J. Enterotoxigenic Potential of Coagulase-Negative Staphylococci. *Int J Food Microbiol*. 2013; 163(1): 34-40.
14. Hu DL, Nakane A. Mechanisms of Staphylococcal Enterotoxin-Induced Emesis. *Eur J Pharmacol*. 2014; 722: 95-107.
15. Otto M. *Staphylococcus aureus* Toxins. *Curr Opin Microbiol*. 2014; 17: 32-7.
16. Ortega E, Abriouel H, Lucas R, Gálvez A. Multiple roles of *Staphylococcus aureus* Enterotoxins: Pathogenicity, Superantigenic Activity, and Correlation to Antibiotic Resistance. *Toxins*. 2010; 2(8): 2117-31.
17. Tranter HS. Foodborne Staphylococcal Illness. *Lancet*. 1990; 336(8722): 1044-6.
18. Wu X, Su YC. Growth of *Staphylococcus aureus* and Enterotoxin Production in Pre-Cooked Tuna Meat. *Food Control*. 2014; 42: 63-70.
19. Wu S, Duan N, Gu H, Hao L, Ye H, Gong W, et al. A Review of the Methods for Detection of *Staphylococcus aureus* Enterotoxins. *Toxins (Basel)*. 2016; 8(7): 176.
20. Da Silva ER, Do Carmo LS, Da Silva N.. Detection of the Enterotoxins A, B, and C Genes in *Staphylococcus aureus* from Goat and Bovine Mastitis in Brazilian Dairy Herds. *Vet Microbiol*. 2005; 106(1-2): 103-7.
21. Schelin J, Wallin Carlquist N, Cohn MT, Lindqvist R, Barker GC, Radstrom P. The Formation of *Staphylococcus aureus* Enterotoxin in Food Environments and Advances in Risk Assessment. *Virulence*. 2011; 2(6): 580-92.
22. Shuiep ES, Kanbar T, Eissa N, Alber J, Lammler C, Zschock M, et al. Phenotypic and Genotypic Characterization of *Staphylococcus aureus* Isolated from Raw Camel Milk Samples. *Res Vet Sci*. 2009; 86(2): 211-5.
23. Hassani S, Hosseini Doust R, Mohebati Mobarez A. Enterotoxin A Gene Barrier *Staphylococcus aureus* within Traditionally Dairy Products of Tehran. *Int J Enteric Pathog*. 2014; 2(4): e20906.
24. Imani Fooladi A, Tavakoli H, Naderi A. Detection of Enterotoxigenic *Staphylococcus aureus* Isolates in Domestic dairy Products. *Iran J Microbiol*. 2010; 2(3): 137-42.
25. Rahbar Saadat Y, Imani Fooladi AA, Shapouri R, Hosseini MM, Deilami Khiabani Z. Prevalence of Enterotoxigenic *Staphylococcus aureus* in Organic Milk and Cheese in Tabriz, Iran. *Iran J Microbiol*. 2014; 6(5): 345-9.
26. Ebrahim Rahimi HM, Amir Shakerian, Hamid Reza Kavyani. The Detection of Classical Enterotoxins of *Staphylococcus aureus* in Raw Cow Milk Using the ELISA Method. *Turk J Vet Anim Sci*. 2012; 36(3): 319-22.
27. Fagundes H, Barchesi L, Filho AN, Ferreira LM, Oliveira CAF. Occurrence of *Staphylococcus aureus* in Raw Milk Produced in Dairy Farms in São Paulo state, Brazil. *Braz J Microbiol*. 2010; 41(2): 376-80.
28. Hwang SY, Kim SH, Jang EJ, Kwon NH, Park YK, Koo HC, et al. Novel Multiplex PCR for the Detection of the *Staphylococcus aureus* Superantigen and Its application to Raw Meat Isolates in Korea. *Int J Food Microbiol*. 2007; 117(1): 99-105.
29. Aragon Alegro LC, Konta EM, Suzuki K, Silva MG, Júnior AF, Rall R, et al. Occurrence of Coagulase-Positive Staphylococcus in Various Food Products Commercialized in Botucatu, SP, Brazil and Detection of Toxins from Food and Isolated Strains. *Food Control*. 2007; 18(6): 630-4.
30. Omoe K, Hu DL, Takahashi-Omoe H, Nakane A, Shinagawa K. Comprehensive Analysis of Classical and Newly Described Staphylococcal Superantigenic Toxin Genes in *Staphylococcus aureus* Isolates. *FEMS Microbiol Lett*. 2005; 246(2): 191-8.