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Molecular detection of enterotoxin genes (*sea* and *sec*) in *Staphylococcus aureus* isolated from dairy products in Karaj, Alborz, Iran

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Abstract. *Staphylococcus aureus,* a significant bacterial agent of food poisoning, particularly the strains producing enterotoxins, is a topic of paramount importance. The prevalence of these enterotoxins in the dairy industry, especially in regions like Karaj city, Alborz province, Iran, with its numerous industrial and traditional dairy companies, is a matter of great concern for public health. This study aims to evaluate the frequency of *sea* and *sec* genes among dairy products in Karaj, Iran. We collected 100 samples of industrial and traditional dairy products, including 25 samples of pasteurized milk, 25 samples of pasteurized ice cream, 25 samples of raw milk, and 25 samples of traditionally made ice cream, throughout Karaj, then transferred them into the laboratory, then cultured on the media containing mannitol salt agar and Brad Parker agar. Second, enzymatic tests, such as catalase and coagulase tests and biochemical and bacterial assessments, including mannitol fermentation and Gram staining methods, were employed to detect *S. aureus* contamination. Besides, the presence of *sea* and *sec* genes was assessed by PCR. Finally, the data were statistically analyzed using SPSS software. The results demonstrated that enzymatic and biochemical methods could detect 54 contaminated samples by *S. aureus* among 100 samples. However, as a reliable molecular technique, PCR detected 57 *S. aureus*-contaminated samples among all the tested samples. Moreover, it is indicated that 12 and 6 PCR-positive samples contained enterotoxin A and C types, respectively.

In brief, the huge amounts of dairy products in Karaj are significantly contaminated by enterotoxin-producing strains of *S. aureus*, especially type A and C.

Keywords: dairy products, enterotoxin A, enterotoxin C, polymerase chain reaction, *Staphylococcus aureus*.

Introduction

Food-borne diseases are considered the most vital issue in public health because millions of people are annually exposed to infections that could lead to hospitalization or death in severe cases. *Staphylococcus aureus* has been known as the second or third microbial agent of infections (Vargová *et al*., 2023), including secondary bacterial pneumonia following respiratory viral infections, bovine mastitis, vein inflammation, meningitis, urinary tract infection (UTI), and local inflammation of the bone endocarditis, and superficial skin lesion (Fabijan *et al*., 2020). *S. aureus* can grow quickly in different conditions, such as mammalian skin surface and mucous membranes. Besides, it can be found in various foods such as dairy, meat, vegetables, salads, and even cooked and salted food (Grispoldi *et al*., 2021). *S. aureus* contains several virulent factors causing pathogenicity and the colonization of different media. Two important virulence factors include bacterial enterotoxins and toxic shock syndrome toxin (TSST), classified as pyrogenic toxin superantigens (PTSAgs), which can significantly influence the host (Hu *et al*., 2021). Superantigens can specifically interact with major histocompatibility complex (MHC) molecules class II, producing extensive lymphocyte T proliferation and damage caused by high-level released cytokines. On the other hand, all the toxin-encoding genes can horizontally be transferred by mobile genetic elements (MGEs) such as phages and pathogenicity islands (PAIs). This could be really dangerous for people at risk, including the geriatric population, offspring, pregnant women, and patients with diabetes or immunocompromised diseases such as acquired immunodeficiency syndrome (AIDS) (Abdurrahman *et al*., 2020).

Staphylococcal enterotoxins can resist high-temperature stress, and the resistance is higher in food than *in vitro* environment. Also, the toxins are biologically active even after heating the food, although pasteurization can remove *S. aureus*. They are also resistant to stomachic and intestinal proteases, so the toxins are still active after digestion (Forouzani-Moghaddam *et al*., 2024). Many factors, such as the number of bacterial cells, salinity (higher than 7.5%), pH, temperature, and competition with other bacteria to achieve more food

resources, influence enterotoxin production in food (Al-Nabulsi *et al*., 2020). It has been demonstrated that there is a close correlation between enterotoxin production in dairy and food poisoning. Actually, 10^5-10^8 cfu/g of *S. aureus* can cause food poisoning (Vargová *et al*., 2023). Many studies have indicated that 15-80% of *S. aureus*, isolated from dairy products such as milk, cheese, ice cream, or even other protein-contained products, can biosynthesize enterotoxins. The symptoms of food poisoning caused by staphylococcal enterotoxins include nausea, vomiting, rarely diarrhea, and muscle and abdominal pain. There are also many significant economic losses from food poisoning caused by enterotoxigenic strains of *S. aureus* (Fabijan *et al*., 2020). This study aimed to evaluate the frequency of *sea* and *sec* genes in *Staphylococcus aureus* isolated from dairy products in Karaj, Alborz, Iran.

Materials and methods

Sampling

In the study, 100 samples of traditional and industrial dairy products, including 25 samples of raw milk, 25 samples of traditionally made ice cream, 25 samples of pasteurized milk, and 25 samples of pasteurized ice cream, were collected throughout Karaj. They were then transferred into the laboratory within sterilized tubes and incubated on mannitol salt agar and Brad Parker agar media at 37 °C for 48 hours. The phenotypic characteristics of the grown colonies were visually assessed.

Microbiological analyses

Biochemical analyses including Gram staining (Beveridge, 2001), fermentation of the mannitol (Lally *et al*., 1985), and culturing on Brad Parker (BP) medium (Parkor and Hewiit, 1970), as well as enzymatic assessment including catalase (Reiner, 2010) and coagulase tests (Sperber and Tatini, 1975), were used to detect *Staphylococcus spp*. and *S. aureus*, respectively. *S. aureus* can reduce potassium tellurite to metallic tellurium on the BP medium to form black colonies (Parkor and Hewiit, 1970).

Polymerase chain reaction

The bacterial DNA was extracted by DNG superscript –Plus kit (CinnaGen Co., Tehran, Iran). Specific primers were designed by Oligo v.7 software and then synthesized for *sea* and *sec* genes and *nuc* gene encoding deoxyribonuclease as an index to detect *S. aureus* (Tab. 1). *S. aureus* colonies were detected by PCR

using specific primers for the *nuc* gene. The positive samples with *S. aureus* were molecularly analyzed by PCR and the specific primers for *sea* and *sec* genes were used. Reference strains (Iranian Biological Resource Center, Tehran, Iran) were used as a positive control in PCR (Tab. 2). Gel electrophoresis (agarose 1.5%) was used to track the amplified DNA fragments.

Statistical analysis

Chi-square and t-tests were employed to statistically analyze the data, derived from PCR and the results from biochemical and enzymatic assessments by SPSS software (version 26.0.0.1).

Table 1. The primers used in the study

Gene	Sequence (5'-- 3')	Primer size (nt)	Product Size (bp)
nuc	F- GCG ATT GAT GGT GAT ACG GTT	21	279
	R- AGC CAA GCC TTG ACG AAC TAA AGC	24	
sea	F- GCA GGG AAC AGC TTT AGG C	19	520
	R- GTT CTG TAG AAG TAT GAA ACA CG	23	
sec	F- CTT GTA TGT ATG GAG GAA TAA CAA	24	283
	R- TGC AGG CAT CAT ATC ATA CCA	21	

Table 2. PCR programs used for amplification

Genes		*nuc*		*sea*		*sec*	
Steps		Temp. (˚C)	Time	Temp. (°C)	Time	Temp. (˚C)	Time
Initial denaturation		95	5 min	95	5 min	95	5 min
37 cycles	Denaturation	95	40 s	95	30 s	95	40 s
	Annealing	55	1 min	58	45 s	58	45 s
	Extension	72	80 s	72	1 min	72	1 min
Final extension		72	10 min	72	7 min	72	10 min

Temp. = temperature.

Results

Biochemical analyses

The results of mannitol fermentation on mannitol salt agar media (Fig. 1) indicated that 60 samples could grow on the media and form some bacterial and yeast colonies. Besides, raw milk contains more bacteria (17 samples), which could cause mannitol fermentation. Although the lowest rate of mannitol fermentation was observed in pasteurized ice cream (4 samples), there was statistically no significant difference between traditional ice cream (6 samples), pasteurized milk (5 samples), and ice cream (Tab. 3).

Interestingly, the number of black colonies was the highest for samples derived from raw milk (17 samples) on the Brad Parker media. Only two samples of pasteurized milk and ice cream formed black colonies, while six samples of traditional ice cream formed black colonies (Tab. 3; Fig 2).

Figure 1. Fermentation of mannitol salt agar

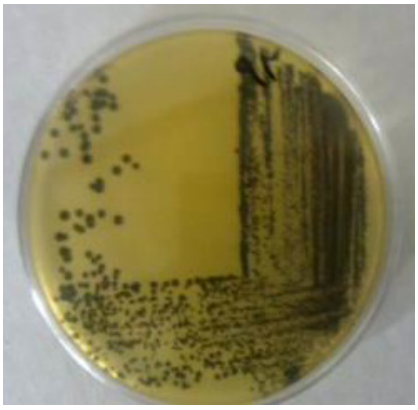

Figure 2. Black colonies formed by *S. aureus*

Table 3. The number of positive samples for each biochemical test

Test	Pasteurized milk	Pasteurized ice cream	Traditional ice cream	Raw milk
Mannitol salt agar	5	4	6	17
Brad Parker agar	2	2	6	17
Gram stain	5	2	8	17

It is also interesting that 17 raw milk samples contained Gram-positive bacteria that were expected to be *S. aureus* and 8, 5, and 2 samples for traditional ice cream, pasteurized milk, and ice cream, respectively (Tab. 1; Fig. 3).

Enzymatic analyses

Each colony grown on mannitol salt agar, suspected to contain *S. aureus*, was used for enzymatic analyses. As per the catalase test results, all 25 samples of raw milk and traditional ice cream were contaminated by *S. aureus*. There were also 23 and 24 *S. aureus*-infected samples for pasteurized milk and ice cream, respectively (Tab. 4).

For the coagulase test, 14 samples of the raw milk were coagulase-positive. In contrast, there was no coagulase-positive sample for pasteurized ice cream. Moreover, 2 coagulase-positive samples for pasteurized milk and 3 coagulase-positive samples for traditional ice cream were observed (Fig. 4; Tab. 4).

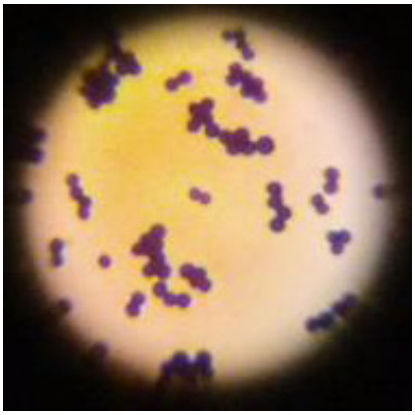

Figure 3. Grape-like clusters (cocci) arrangement of *S. aureus*

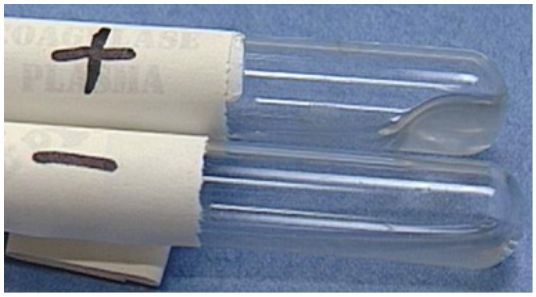

Figure 4. Positive and negative samples for the coagulase test

Table 4. The number of positive samples for each enzymatic test

Test	Pasteurized milk	Pasteurized ice cream	Traditional ice cream	Raw milk
Catalase	23	24	25	25
Coagulase	2	0	3	14

PCR

PCR by specific primer for the *nuc* gene detected 57 *S. aureus*-infected samples among all 100 samples. Also, using specific primers for *sea* and *sec* genes could repectively detect 11 and 6 many samples containing the genes encoding enterotoxins A and C on those 57 positive samples (Fig. 5). Details of the PCR test for each sample of the dairy products are in Tab. 5.

Table 5. The number of Positive-PCR samples for each gene

Gene	Pasteurized milk	Pasteurized ice cream	Traditional ice cream	Raw milk
nuc	17	7	14	19
sea	1	3	1	7
sec	0	2	1	3

Statistical analysis

The chi-square test of independence showed a significant difference between biochemical and enzymatic tests and PCR detection ($p<0.05$). The detection of *S. aureus* in samples by PCR containing specific primers for the nuc gene showed significant differences between pasteurized and traditional ice creams ($p<0.05$) but not between raw and pasteurized milk ($p>0.05$; Tab. 6).

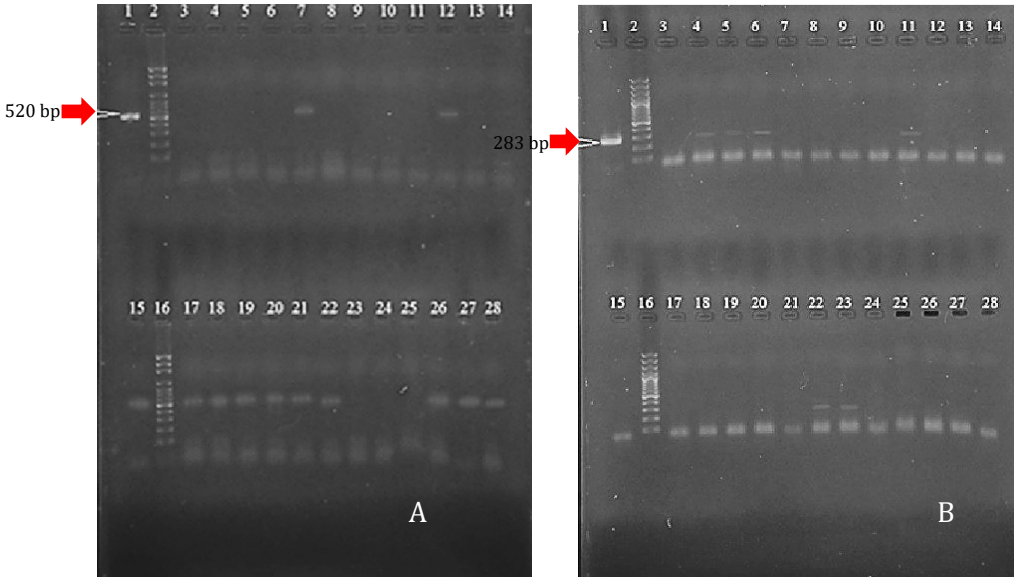

Figure 5. Gel electrophoresis of the PCR products, genes encoding: A) Enterotoxin A, 1: C+, 2 & 16: DNA molecular ladder, 3: C-, 4-8: Pasteurized milk, 9-14: Traditional ice cream, 15,17-22: Raw milk, 23-28: Pasteurized ice cream; B) Enterotoxin, 1:C+, 2 & 16: DNA molecular ladder, 3-7: Raw milk, 8-14: Traditional ice cream, 15: C-, 17-21: Pasteurized milk, 22-28: Pasteurized ice cream.

Table 6. Chi-Square Test for comparing biochemical and enzymatic tests and PCR

Statistical test	Value	df	P value	Exact Sig.	
				(2-sided)	(1-sided)
Pearson Chi-Square	11.636 a	1	.001		
Continuity Correction*	10.687	1	.001		
Likelihood Ratio	11.757	1	.001		
Fisher's Exact Test				.001	.001
Linear-by-Linear Association	11.578	1	.001		
N of Valid Cases	200				

a. 0 cells (0%) have an expected count of less than 5. The minimum expected count is 45.00. * Computed only for a 2x2 table

For detecting enterotoxin A using PCR containing specific primers for the *sea* gene, there is a significant difference among all samples derived from any studied dairy products ($p<0.05$). The difference is insignificant for the *sec* gene at the 5% level, while there is a difference between samples (Tab. 7).

Table 7. Chi-Square Test for PCR results

Bacteria	Statistical test	Value	Df	P value
Staphylococcus	Pearson Chi-Square	13.505 b	3	.004
	Likelihood Ratio	13.821	3	.003
	Linear-by-Linear Association	3.563	1	.059
	N of Valid Cases	100		
Enterotoxin A	Pearson Chi-Square	9.091 c	3	.028
	Likelihood Ratio	8.597	3	.035
	Linear-by-Linear Association	.300	1	.584
	N of Valid Cases	100		
Enterotoxin C	Pearson Chi-Square	3.546 d	3	.315
	Likelihood Ratio	4.712	3	.194
	Linear-by-Linear Association	.140	1	.708
	N of Valid Cases	100		
Total	Pearson Chi-Square	11.289 a	3	.010
	Likelihood Ratio	10.953	3	.012
	Linear-by-Linear Association	1.875	1	.171
	N of Valid Cases	300		

a. 0 cells (0.0%) have an expected count of less than 5. The minimum expected count is 18.75.
b. 0 cells (0.0%) have an expected count of less than 5. The minimum expected count is 10.75.
c. 4 cells (50.0%) have an expected count of less than 5. The minimum expected count is 3.00.
d. 4 cells (50.0%) have an expected count of less than 5. The minimum expected count is 1.50.

Discussion

S. aureus can be found as a resident bacteria on the skin and mucus of humans and animals, which are the initial resources of the bacteria. So, they can be transferred to the food during preparing, providing, packing, and storing food (Grispoldi *et al*., 2021). They can be found in a variety of foods, such as meat products, milk, and dairy products, fruit juice, as some proper media for the growth of the bacteria because they can grow independently in any conditions (Forouzani-Moghaddam *et al*., 2024). Notably, a lack of personal and environmental hygiene can facilitate food contamination by *S. aureus*, and consequently cause food poisoning (Vargová et al., 2023).

According to the universal standard, first-grade milk contains less than 100,000 microorganisms per ml. Also, the microbial load of milk should not be more than 750,000 microorganisms per ml (Tilocca *et al*., 2020; U.S. Department of Health and Human Services, Public Health Service and Food and Drug Administration, 2017). Although standard milk is retained in inappropriate conditions, *S. aureus* can quickly grow at the proper temperature and duration for bacterial growth and then infect the milk. Thus, the microbial load can be enhanced (El-Mokadem *et al*., 2020). If the milk is contaminated by *S. aureus*, pasteurization will be able to remove the bacteria; then, there is no bacterial risk to consumers' health. However, *S. aureus*, producing enterotoxin, could threaten consumers by causing food poisoning if the bacteria produced the toxins before pasteurization (Almutawif *et al*., 2019). Enterotoxins, particularly encoded by *sea* and *sec* genes, are the most crucial factors causing food poisoning (Forouzani-Moghaddam *et al*., 2024).

Staphylococcal enterotoxins can be detected by many important methods, including standard immunological techniques (Nouri *et al*., 2018). However, there are some disadvantages to using the methods, such as the long time needed to prepare the right conditions to produce toxins by the bacteria, cross-reactivity, and the probability of false results (Ali *et al*., 2020). On the other hand, the PCR test can detect enterotoxin-producing bacteria, even though the genes encoding the enterotoxins are not expressed. Thus, PCR could effectively detect *S. aureus*-producing enterotoxins A and C as the most crucial and common factors in food poisoning (Osman et al., 2020). Low concentrations of these types of enterotoxins can cause severe food poisoning. Thus, PCR is commonly used to detect enterotoxin-encoding genes. Many studies have used PCR to detect staphylococcal enterotoxins (Forouzani-Moghaddam *et al*., 2024). For example, Osman *et al*. utilized PCR to detect *S. aureus* and enterotoxins in buffalo milk (Osman *et al*., 2020).

Moreover, PCR detected the bacteria producing enterotoxins within milk and Coalho cheese in Brazil (Pereira *et al*., 2018). Wang *et al*. (2019) used PCR to detect *S. aureus* causing bovine mastitis and subsequent milk contamination. Finally, the molecular technique was employed to diagnose milk contamination by *S. aureus* and encode the gene for enterotoxin C in raw milk from several dairy farms in Java, Indonesia (Harijani *et al*., 2020).

In the present study, statistical analysis indicated that PCR is a significantly more precise technique than biochemical and enzymatic methods for diagnosing *S. aureus*-contaminated dairy products and detecting *S. aureus*-producing enterotoxins A and C.

Conclusions

According to the heat resistance of enterotoxins A and C, *S. aureus*-contaminated dairy products can be toxic and cause gastroenteritis in a short period even though they are heated. Therefore, Detecting genes encoding enterotoxins via PCR, a specific, sensitive, fast, and inexpensive molecular method, could be used instead of the conventional immunological assay techniques.

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