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ORIGINAL ARTICLE

Detection of genes encoding enterotoxins (SEA-D) in *Staphylococcus aureus* strains isolated from workers' nasal samples and creamy pastries of Shiraz confectioneries

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ABSTRACT

Background and Objectives: *Staphylococcus aureus* is responsible for the majority of food poisoning all around the world. Nasal carriers of *S. aureus* and foodstuffs need for handling are important sources and vehicles to transmit this pathogen to ready-to-eat foods. According to hygienic standards, confectioners should not be contaminated with *S. aureus.* This study aimed to detect nasal carriers and creamy pastries contaminated with enterotoxigenic *S. aureus* in confectioneries of Shiraz, Iran.

Materials and Methods: From the confectioneries of Shiraz city, 27 places in the north, south, center, west, and east areas were selected randomly then 100 creamy pastries samples and 117 nasal swabs were collected. Bacteriological and biochemical tests were performed to isolate *S. aureus.* The polymerase chain reaction (PCR) test was used to identify the virulence and enterotoxins genes of the *S. aureus* isolates. Agar disk diffusion was performed to find out the antibiotic resistance of the isolates.

Results: Results revealed that 16.24 and 33 percent of workers and creamy pastries were contaminated with *S. aureus* respectively. Also, 100%, 37%, 58%, and 6% of nasal samples harbored *femA*, *mecA*, *sea*, and *sec* genes respectively. According to the results 97%, 70%, 54.5%, and 6% of creamy pastries isolates harbored *femA*, *mecA*, *sea*, and *sec* genes respectively. No isolate carried *seb* and *sed* genes. The results also showed that 41.5% of nasals and 5.5% of creamy pastry isolates harbored both *sea* and *sec* genes. The *sea* was the most common enterotoxin gene observed in nasal and creamy pastries. The results of the antimicrobial resistance test showed that 68.42% of nasal and 48.48% of creamy pastry isolates were resistant to cefoxitn (FOX) respectively. Both nasal (89%) and creamy pastry (82%) isolates presented the highest resistance to penicillin (P) and the most sensitivity to trimethoprim-sulphamethoxazole (SXT) (94%). Most of the isolates were sensitive to erythromycin (E), aztreonam (AZM), tetracycline (TE), trimethoprim (TMP), and ciprofloxacin (CP). Isolates of *S. aureus* harboring multi-enterotoxin genes were resistant to more antibiotics than others.

Conclusion: The presence of enterotoxigenic *S. aureus* in the workers' nasal samples and creamy pastries of Shiraz confectioneries was high which is a potential public health hazard.

Keywords: *Staphylococcus aureus*; Creamy pastry; Enterotoxin genes; Antibiotic resistance

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INTRODUCTION

Consuming foodstuffs contaminated with pathogens and their toxins is responsible for hospitalizing and economic losses (1). Food preparation and distribution can be involved in the occurrences of foodborne illnesses contaminated with various pathogens such as *Staphylococcus aureus* (2, 3).

S. aureus is non-motile, facultatively anaerobic, Gram-positive cocci, mannitol utilization, thermostable nuclease "D-Nase, catalase, and coagulase positive. The organisms can grow in a wide range of temperatures and pH also sodium chloride concentrations (up to 15%). *S. aureus* survives in a wide variety of foods; especially those that require considerable handling during preparation. Humans and animals are primary reservoirs of *Staphylococcus* and this organism is present in the nasal, throat, hair, and skin of probably 20-30 percent of healthy individuals. *S. aureus* is one of the most common pathogens that produce staphylococcal enterotoxins (SEs) in food and consequently result in foodborne poisoning (4, 5). Studies showed that the handling of cream-filled pastries in the procedure of production makes food incriminated in staphylococcal intoxication, potentially (6).

According to the antigenicity (sequence homology), 24 different SEs and based on serological groups 6 groups (staphylococcal enterotoxin types A, B, C, D, E, and H) have been discovered. The most occurrence of enterotoxins production by *S. aureus* is related to SEA and SEB (7).

One of the most important and greatest worldwide challenges in medicine is antibiotic resistance which is a silent global crisis. Strains of multi-drug resistance such as *S. aureus* are found in developed and underdeveloped countries (8, 9).

Creamy pastries are ready-to-eat and produced in the confectioneries that are popular in Iran. Nasal and hand carriers of *S. aureus* can contaminate the products and result in intoxication in consumers. This study aimed to detect antibiotic resistance and some gene encoding enterotoxins (SEA-D) of *S. aureus* from creamy pastries and workers' nasal in Shiraz, Iran.

MATERIALS AND METHODS

Sampling. A hundred creamy pastries and 117 nasal swabs of the workers from 27 confectioneries in different parts of Shiraz city were collected randomly. All samples were stored in the cold box and transferred to the laboratory rapidly.

Detection of *S. aureus*. For the detection of *S. aureus* in the creamy pastries, 25 grams of the samples were added to 225 mL of ringer at a sterile condition and thoroughly mixed gently. One milliliter of suspension was added to the TSB tube and incubated at 37°C for 24 hours. Then 0.1 mL of microbial suspension was stroked on Baird Parker agar and incubated for 24 to 48 hours.

Nasal samples were cultured on a blood agar medium enriched with defibrinated ox blood and incubated at 37°C for 24 hours to determine the hemolytic activity of the isolates. Each colony with a hemolytic zone was incubated in a TSB tube and then cultured on Baird Parker agar.

Suspected colonies on Baird Parker agar (shiny and black with lecithin lysis) were selected for confirmation tests. Conventional biochemical tests (catalase activity, hemolysis, mannitol fermentation, coagulase, thermostable nuclease "DNase activity) and Gram staining were performed to the identification of *S. aureus* (10).

DNA extraction. A single pure colony of *S. aureus* was cultured overnight in a TSB tube at 37°C. The genomic DNA of the isolates was extracted using a Genomic DNA Purification Kit (Sinaclon, Iran) according to the manufacturer's protocol. Extracted DNA was quantified at 260/280 nm by spectrophotometer (Nanodrop, Germany).

PCR amplification. Reference primers (Table 1) were used to amplify *mecA, femA,* and *sea-d* genes.

The PCR reaction volume was prepared for 25 µl consisting of 12.5 µl 2× PCR master mix, 1 µl forward primer, 1 µl reverse primer, 8.5 µl nuclease-free water, and 2 µl of DNA templet.

The PCR reaction conditions were as follows: initial denaturation at 94°C, 1 cycle for 4 min; denaturation at 94°C for 1 min; annealing temperature (variable for each primer in Table 1) for 40 s; extension at 72°C for 2 min for 35 cycles; and a final extension at 72°C for 10 min. The amplicons were visualized using 1% agarose gel.

Antimicrobial susceptibility test. Isolated *S. aureus* was subcultured into TSB and incubated at 37°C

Table 1. Primers planned to determine genes encoding enterotoxins in *S. aureus*

Target Genes	Oligonucleotide sequence (5'-3')	Size (bp)	Annealing Temperature (°C)
femA	F 5′-CATGATGGCGAGATTACAGGAT-3′	372	55
	R 5′-CGCTAAAGGTACTAACACACGG-3′		
mecA	F 5' GTGAAGATATACCAAGTGATT3'	147	50
	R 5'ATGCGCTATAGATTGAAAGGAT3'		
sea	F 5′-CCTTTGGAAACGGTTAAAACG-3′	127	55
	R 5′-TCTGAACCTTCCCATCAAAAAC-3′		
seb	F 5' TCG CAT CAA ACT GAC AAA CG 3'	477	56
	R 5'AGG TAC TCT ATA AGT GCC TGC CT 3'		
sec	F 5' CTC AAG AAC TAG ACA TAA AAG CTA GG 3'	271	51
	R 5' TTA TAT CAA AAT CGG ATT AAC ATT ATC 3'		
sed	F 5' CTA GTT TGG TAA TAT CTC CTT TAA ACG 3'	319	53
	R 5'TTA ATG CTA TAT CTT ATA GGG TAA ACA TC 3'		

for 4-6 hours. Microbial suspensions were prepared in 0.5 McFarland's concentration. Also, *S. aureus* ATCC 25923 was prepared in the same concentration as the positive control. The tests were performed using the disc diffusion method on Muller Hinton agar using cefoxitin (FOX-30 µg), ciprofloxacin (CP-5 µg), azithromycin (AZM-15 µg), erythromycin (E-15 µg), tetracycline (TE-30 µg), penicillin (P-10 U), trimethoprim (TMP-5 µg), cefoxitin (FOX 30 µg) and trimethoprim-sulfamethoxazole (SXT-1.25/23.7 µg) recommended by the Clinical Laboratory Standards Institute (CLSI) guidelines (11). The inhibition zone of the isolates was classified as CLSI charts into 3 groups sensitive, intermediate, and resistant.

Data analysis. Data were statistically analyzed using SPSS Software version 26. Chi-square and Fisher's exact tests were performed and $P < 0.05$ was determined as significant.

Ethics statement. The experimental procedures and protocols used in this study were approved by the Animal and Human Ethics and Welfare Committee of Shahrekord University (approval number IR.SKU.AC.REC1400.010).

RESULTS

Microbiological and Biochemical tests identified that 18.8 and 44 percent of workers' nasal and creamy pastries samples were contaminated with coagulase-positive staphylococci, respectively. PCR tests revealed that 95 percent of coagulase-positive staphylococci of nasal and 75 percent of creamy pastries isolates harbored *mecA* and *femA* genes that were determined as *S. aureus* (Table 2).

Table 3 presents the prevalence of enterotoxin genes (*sea-d*) among coagulase-positive staphylococcus strains (*S. aureus* and none *S. aureus*) isolated from workers' nasal swabs and creamy pastries. All *S. aureus* strains isolated from nasal and 97 percent of creamy pastries harbored the *femA* gene. But the prevalence of the *mecA* gene among the nasal and creamy pastries was 37 and 70 percent, respectively. Results also showed that all staphylococci strains have not contained *seb* and *sed* genes. From 3 nasal non-aureus isolates, just one isolate harbored *SEA* and *SEC* genes and the others had no gene. *sea* gene was the most common enterotoxin gene observed in the nasal and creamy pastries products (Fig. 1). The results showed that 5 nasals and 2 creamy pastry isolate harbored both *esa* and *esc* genes.

Table 4 shows that some *S. aureus* strains isolated from workers' nasal and cream pastries of Shiraz confectioneries harbor more than two genes (multiple genes). Data showed that 7 (36.84%) and 12 (36.36%) of *S. aureus* strains isolated from nasal and creamy pastries were carrying multi-virulence genes respectively (Table 4).

The results of the antimicrobial resistance tests on *S. aureus* isolates have shown in Table 5. The highest resistance of the isolates was related to penicillin. All isolates presented the highest sensitivity to trimethoprim-sulfamethoxazole.

Multiple antibiotic resistance patterns were observed

Table 2. Frequency of biochemical tests performed on workers' nasal and creamy pastries isolates.

Samples	Gram +	Catalase +	Mannitol fermentation +	DNase +	Coagulase +	Coagulase + (Harboring *mecA* and *femA* genes)
Nasal (n=117)	114 (97.44%)	112 (95.73%)	24 (20.51%)	25 (21.37%)	22 (18.8%)	19 (16.24%)
Creamy pastry (n=100)	50 (50%)	50 (50%)	40 (40%)	35 (35%)	44 (44%)	33 (33%)

Table 3. Prevalence of some virulence genes among coagulase-positive *Staphylococcus* isolates in workers' nasal and creamy pastries samples of Shiraz confectioneries.

Samples	*Staphylococcus aureus*	Enterotoxin genes %					
		femA	*mecA*	*sea*	*seb*	*sec*	*sed*
Nasal	19	100	37	58	0	6	0
Creamy pastry	33	97	70	54.5	0	6	0

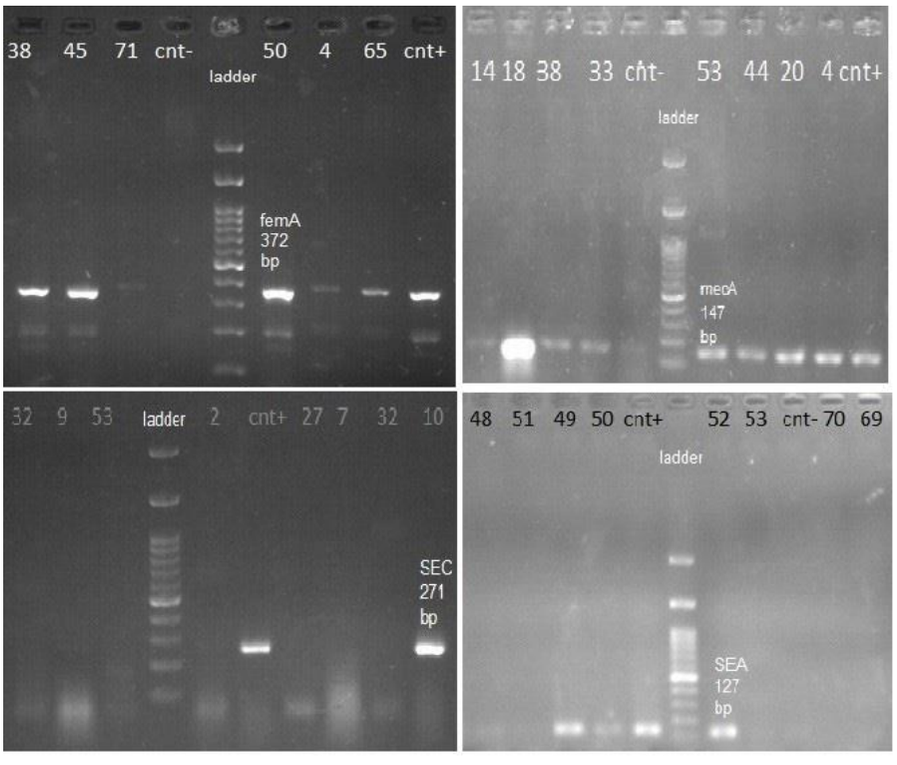

Fig. 1. Entrotoxine genes of *S. aureus* strains isolated from nasal and creamy pastries of Shiraz confectioneries. Cnt-: negative control, Cnt+: positive control (*S. aureus* ATCC 25923), Ladder: 100 bp

among *S. aureus* isolates (Table 6). According to the results, 71.43% of nasal isolates and 58.33% of creamy pastry isolates that harbored multi-virulence genes, were resistant to two or more antibiotics. The statistical test showed that there is a significant relationship between the number of enterotoxin genes and antibiotic resistance in the isolates ($P<0.05$).

DISCUSSION

According to the national standard of Iran, creamy pastries should not be contaminated with *S. aureus* (10). Our results identified that 16.24 and 33 percent of workers' nasal and creamy pastries were contaminated with *S. aureus*, respectively. Other studies in

Table 4. Frequency of harboring multiple genes by *S. aureus* isolated from workers' nasal and cream pastries of Shiraz confectioneries.

	Genes	Isolates
Nasal samples (n=7)	*femA, sea sec femA,*	4
	mecA, sea sec femA,	1
	mecA sea femA,	1
	mecA sec femA,	1
Creamy pastries	*mecA, sea sec femA,*	2
samples (n=12)	*mecA, sea femA,*	9
	mecA, sea	1

Iran have been revealed that approximately 20 to 30 percent of creamy pastries contaminated with this pathogen (12, 13). Sami et al. (2013) showed that 20 percent of the cream-filled sweets offered in the confectioneries of Kerman were contaminated with *S. aureus* (14). Another study in Iran showed that 31 percent of the creamy pastries were infected with *S. aureus* (15). Aycicek et al. (2004) studied the prevalence of *S. aureus* in ready-to-eat meals of military canteens in Ankara, Turkey. Out of 512 samples, 48 samples (9.4%) were contaminated with coagulase-positive staphylococci (16).

Table 5. Antibiotic resistance pattern of *S. aureus* isolated from nasal and creamy pastries samples of Shiraz confectioneries.

Isolates	Type of antibiotic	S (%)	I (%)	R (%)
Nasal (n=19)	Cefoxitin (FOX)	31.58	21.05	47.37
	Erythromycin (E)	52.63	42.11	5.26
	Aztreonam (AZM)	89.47	5.26	5.26
	Tetracycline (TE)	78.95	0	21.05
	Penicillin (P)	10.53	0	89.47
	Trimethoprim (TMP)	91.74	0	8.26
	Ciprofloxacin (CP)	82.21	15.79	0
	Trimethoprim Sulphamethoxazole (SXT)	94.74	5.26	0
Creamy pastry (n=33)	Cefoxitin (FOX)	51.51	15.15	33.33
	Erythromycin (E)	69.7	27.3	3
	Aztreonam (AZM)	84.8	6.1	9.1
	Tetracycline (TE)	84.8	0	15.2
	Penicillin (P)	18.2	3	78.8
	Trimethoprim (TMP)	90.9	3	6.1
	Ciprofloxacin (CP)	81.8	18.2	0
	Trimethoprim Sulphamethoxazole (SXT)	93.9	0	6.1

S: sensitive, I: intermediate, R: resistance

Table 6. Multiple antibiotic resistance patterns among *S. aureus* strains harboring multi-virulence genes isolated from nasals and creamy pastries of Shiraz confectioneries.

Samples	Type of Antibiotics	Isolates (%)
Nasal (n=7)	Penicillin, erythromycin, tetracycline, and cefoxitin	1 (14.28)
	Penicillin, aztreonam, erythromycin, ciprofloxacin, and cefoxitin	1 (14.28)
	Penicillin, tetracycline, and cefoxitin	1 (14.28)
	Penicillin, aztreonam, erythromycin, and cefoxitin	1 (14.28)
	Erythromycin and cefoxitin	1 (14.28)
Creamy pastry (n=12)	Penicillin and tetracycline	1 (8.33)
	Penicillin, aztreonam, and erythromycin	1 (8.33)
	Penicillin and cefoxitin	5 (41.66)

S. aureus may contaminate confectionery products via nasal carriers and cross-contamination by food handlers, food contact surfaces, and initial ingredients of sweets. Therefore, the prevalence and occurrence of the bacterium in creamy pastries are different in various regions.

The results of the present study showed that all *S. aureus* isolates from nasal samples and 97 percent of creamy pastries harbored the *femA* gene. Also, 37 and 70 percent of *S. aureus* from nasal samples and creamy pastries presented methicillin resistance genes (*mecA*), respectively. The prevalence of *sea* and *sec* genes were 58 and 6 percent in nasal isolates and 54.5 and 6 percent in creamy pastries, respectively. There were no identified other enterotoxin genes in *S. aureus* isolates.

Rezaei (2018), determined only the sea gene in 26 percent of creamy sweets *S. aureus* isolates (17). Asgarpour et al. (2018) isolated *S. aureus* from 46 nasal swabs of workers at the butcher, dairy stores, and restaurants in Ardabil city. The presence of genes encoding *sea*, *seb*, and *sec* in isolates was 23.9, 13, and 10.8 percent, respectively (18). There are some reports of contamination of hands and nasal mucosa of people who deal with food with *Staphylococcus aureus* containing enterotoxin genes, and this issue has become an important health problem. (19).

Unlike other studies, Machado et al. (2020) showed that a high percentage of *S. aureus* isolated from food, staff, and surfaces, harboring *seg* and *see* genes (20).

Regarding the results of the present study, a high percentage of *S. aureus* isolates were resistant to cefoxitin and penicillin. But, according to the study by Soltan Dallal et al. (2020), 5.2, 5.2, and 23.1 percent of the isolated *S. aureus* from creamy pastries were resistant to erythromycin, ciprofloxacin, and tetracycline, respectively (21).

Our study also showed that *S. aureus* isolates carrying the enterotoxin gene were more resistant to a variety of antibiotics. Multi-enterotoxin genes and -multi-antibiotic resistance isolates were observed among both nasal and creamy pastries samples collected from Shiraz confectioneries. Analysis of the relation of antibiogram tests and PCR results certified that isolates of *S. aureus* harboring multi-enterotoxin genes were resistant to more antibiotics than others.

Mirzababaei et al. (2021) identified that 17.6 % of samples from food outbreaks cases were contaminated with *S. aureus* and indicated that most of the isolates were resistant to penicillin (22). In another study, Hashemi et al. (2020), showed that all enterotoxigenic *S. aureus* isolated from creamy pastries were resistant to penicillin and 95 percent of isolates harboring the *sea* gene were resistant to erythromycin and ciprofloxacin (23). Jassim et al. (2021) also reported that 75 percent of multi-resistant *S. aureus* isolated from cakes harbored enterotoxin genes (24).

High-level resistance to methicillin is caused by the *mecA* gene, which encodes an alternative penicillin-binding protein, PBP 2a, also, the *femA* gene, a cytoplasmic protein necessary for the expression of methicillin resistance in *S. aureus* and also involved in the biosynthesis of staphylococcal cell walls. The results of the present study indicate 37 percent of nasal and 70 percent of creamy pastries also, 100% of nasal and 97% of creamy pastries isolates harbored *mecA* and *femA* genes, respectively. In other studies, the resistance of *S. aureus* isolated from food to methicillin has been reported (25, 26). Methicillin-resistant *S. aureus* isolated from ready-to-eat food samples in developing countries was reported by Aminul Islam et al. (27).

CONCLUSION

The presence of *S. aureus* in creamy pastries is a crisis and a potential hazard to food safety. The prevalence of *S. aureus* isolated from workers' nasal samples and creamy pastries was generally high. The isolates harbor some virulence genes incriminated in food poisoning and antibiotic resistance. Training food safety to workers and further supervision by the local health authorities are required to reduce the contamination levels.

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