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ORIGINAL ARTICLE **OPEN ACCESS**

Evaluating the presence of enterotoxin genes in *Staphylococcus aureus* isolates recovered from human nasal carriers

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ABSTRACT
A total of 40 Staphylococcus aureus strains isolated from nasal carriers, were analyzed for the presence of the enterotoxin genes sea, seb, seg, seh, sei and sej using Multiplex PCR. 22.5% of isolates were positive for one or more toxin genes. The seg gene was found most frequently (17.5%), followed by seh (12.5%), sea (10%), sei 10%), sej (10%) and finally, seb (5%).Our results revealed that Multiplex PCR method is simple, sensitive, low cost, relatively rapid and very specific; In addition, it can identify several genes that encode toxin at the same time. Besides, the results of this study showed that the presence of S. aureus in the human anterior nares in general, and enterotoxigenic strains, in particular, can be a potential risk for health.
Keywords*: Staphylococcus aureus, enterotoxin genes, Multiplex PCR*

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INTRODUCTION

Staphylococcus aureus is one of the major bacterial pathogens which cause serious infections in humans, including endocarditis, pneumonia, toxic shock syndrome, and bacteraemia [29].
Some strains of this organism can also be the pathogen responsible for food poisoning by production of enterotoxins when growing in foods [22].
Staphylococcal enterotoxins (SEs) are a group of single-chain, low-molecular weight proteins (MW, 26900–29600Da), that are very resistant to heat and gastrointestinal proteases such as pepsin, that justifies why it remains active after thermal processing of food and ingestion [7, 9, 21, 25]. SEs are usually divided into the classic (SEA to SEE) and newly described (SEG to SER and SEU) enterotoxins [7].
The genes responsible for encoding SEs are often embedded in mobile genetic elements, such as prophages, transposons, plasmids and pathogenicity islands [11], which may be transferred horizontally between staphylococcal strains. So, enterotoxin genes may play an important role in the evolution of *S. aureus* as a pathogen [29].
For the above-mentioned reasons, the evaluation of the enterotoxin genes in *S. aureus* isolates can be useful for epidemiological tracing and evolutionary analysis.
There are various methods for the detection of enterotoxigenic bacteria, such as latex agglutination, ELISA, immunodiffusion and RIA. But these methods are not reliable because specific circumstances are necessary for enterotoxin gene expression. However, despite the presence of entrotoxin genes, in specific circumstances, *S.aureus* may not have the ability to produce toxin which would lead to negative results [26].
In addition, molecular techniques such as PCR and Multiplex PCR are recommended for the detection of *S. aureus* enterotoxin genes [9]. These techniques are rapid, sensitive, specific, and reliable compared to immunological toxin production assays [23]. Another advantage of molecular methods is that strains producing low levels of enterotoxin could be identified by these methods [26]. Therefore, they constitute very valuable tools for routine applications [23].
Besides, *S. aureus* is a ubiquitous bacterium [27]. In humans, the primary habitat of *S. aureus* is the mucous membrane of the nasopharynx, where it exists as a persistent or transient member of the normal microbiota without causing any symptoms [10].
Organisms present in nose can be transmitted into manually handled foods via respiratory secretions or direct contact with hands during manufacturing and handling of food products [8]. *S. aureus* nasal carriage is established constantly in 30-50% of human population [8].Therefore, nasal carriage of *S. aureus* is in

general, and enterotoxigenic strains, in particular, are important sources for the contamination of foods [10].

In this work, Multiplex PCR method was used to evaluate the presence of enterotoxin genes *sea*, *seb*,*seg*, *seh*, *sej*, and *sei* in *S. aureus* strain isolates recovered from human nasal carriers in Ardabil, a Northwest provincial city in Iran.

MATERIAL AND METHODS

Bacterial strains

This study used 40 *S. aureus* strains originating from nasal carriers that were collected and identified by biochemical tests in a previous study .

Isolates were subcultured on nutrient agar (NA) and identified by biochemical tests, which included catalase, coagulase, mannitol fermentation, and hemolysin, DNase, lecithinase, protease, and lipase production [4, 7].

DNA extraction

Bacterial DNA was extracted from overnight broth cultures of the various strains according to the method reported by Atashpaz et al. [2] with some modification.

The DNA extraction process was carried out as follows:

1) One milliliter overnight culture at 37°C in 5ml brain heart infusion (BHI) broth of each strain was transferred into a centrifuge tube and centrifuged at 3500 rpm for 10 min.

2) The supernatant was discarded and the pellet was suspended in 800 µl lysing buffer (2% CTAB (Merck, Hohenbrunn, Germany), 100 mM Tris-HCl (Merck, Darmstadt, Germany), 1.4 M NaCl (Merck, Darmstadt, Germany), 1% PVP (AppliChem, Darmstadt, Germany), 20 mM disodium salt of ethylenediaminetetraacetic acid (Na2EDTA; Merck, Darmstadt, Germany), 0.2% LiCl (Merck, Darmstadt, Germany). The pH was adjusted at ~8 for the solution used before autoclaving and mixed thoroughly. Afterwards, the prepared sample was transferred to a 1.5 ml centrifuge tube.

3) The sample was incubated at 65°C for 30 min and gently shacked every 10 min.

4) The sample was centrifuged at 10000 rpm for 5 min at 4°C.

5) The supernatant was transferred into a new tube and an equal amount of chloroform– isoamylalchol (Merck, Darmstadt, Germany) (24 : 1 vol/vol) was added. Then the tube was gently flipped several times.

6) The sample was centrifuged at 12000 rpm for 8 min at 4°C. The upper phase was then transferred into a new tube.

7) An equal volume of cold (–20°C) isopropanol (Merck, Darmstadt, Germany) was added to precipitate the DNA. Then the sample was stored at –20°C for 30min.

8) The sample was precipitated at 14000 rpm for 10 min at 4°C.

9) For the first washing step, after the removal of the supernatant, 500 µl of 96% ethanol (Merck, Darmstadt, Germany) (4°C) was added to the sample which was then centrifuged at 12000 rpm for 5 min.

10) For the second washing step, the supernatant was removed and 500 µl of 70% ethanol (4°C) was added to the sample which was then centrifuged at 12000 rpm for 5 min.

11) The supernatant was removed and the pellet was dried at room temperature.

12) The genomic DNA pellet was dissolved in 50 µl TE buffer [10 mM Tris-HCI (pH 8.0), 1 mM EDTA (pH 8.0), (Merck, Darmstadt, Germany)] and DNA solution was stored at –20°C.

Assessing the quantity and quality of extracted DNA

The quantity of the extracted DNA was checked by NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Then the A260/A280 absorbance ratio was used to determine undesired contaminations.

To evaluate the quality and intactness of the extracted DNA , gel electrophoresis was carried out by loading 5 µl of extracted DNA on 1% agarose gel (Invitrogen, California, USA), which contained ethidium bromide (1 µg/ml) for DNA staining. For image acquisitions, a G:Box™ gel documentation system (Syngene, Cambridge, United Kingdom) was used.

PCR primer design

Six PCR primers sets were used to detect the staphylococcal enterotoxin A gene (*sea*), staphylococcal enterotoxin B gene (*seb*), staphylococcal enterotoxin G gene (*seg*), staphylococcal enterotoxin H gene (*seh*) , staphylococcal enterotoxin I gene (*sei*) and staphylococcal enterotoxin J gene (*sej*) as primers reported previously in literature (table 1).

The primers used in this study and their respective amplified products are listed in Table 1.

Table 1. PCR primers sets were used to detect genes *sea*, *seb*, *seg*, *seh*, *sei* and *sej*

Gene	Primer name	Primer sequences	Size of amplified product	references
sea	SEA-f	GCA GGG AAC AGC TTT AGG C	521bp	[16]
	SEA-r	GTT CTG TAG AAG TAT GAA ACA CG		
seb	SEB-f	ACA TGT AAT TTT GAT ATT CGC ACT G	667 bp	[14]
	SEB-r	TGC AGG CAT CAT GTC ATA CCA		
seg	SEG-1	AAG TAG ACA TTT TTG GCG TTC C	287 bp	[19]
	SEG-2	AGA ACC ATC AAA CTC GTA TAG C		
seh	SEH-f	CAA CTG CTG ATT TAG CTC AG	360bp	[16]
	SEH-r	GTC GAA TGA GTA ATC TCT AGG		
sei	SEI-1	GGT GAT ATT GGT GTA GGT AAC	454bp	[19]
	SEI-2	ATC CAT ATT CTT TGC CTT TAC CAG		
sej	SEJ-1	CAT CAG AAC TGT TGT TCC GCT AG	142bp	[16]
	SEJ-2	CTG AAT TTT ACC ATC AAA GGT AC		

Multiplex PCR amplification

In the recent study, genomic DNA of *S. aureus* strains was amplified in one set of Multiplex PCR.

Multiplex PCR reaction was performed in a 25 µL volume, consisting of: 12.5 µL PCR Master Kit (2X), (Cinagen Inc., Tehran, Iran), 0.4 µM; of each primers and 50 ng/µl genomic DNA.

The amplification program consisted of one initial denaturation at 94°C for 4 min followed by 32 cycles of 50 sec at 94°C for denaturation 40 sec at 56°C for primer annealing, 180 sec at 72°C for extension and DNA synthesis and final extension at 72°C for 10 min. The products were separated on 1.5% agarose gel containing ethidium bromide (1 µg/ml), then images were taken using a G:Box™ gel documentation system (Syngene, Cambridge, United Kingdom).

RESULTS

In the present study, 40 *S. aureus* strains originating from nasal carriers were tested for enterotoxin production by Multiplex PCR assay. Testing with specific primers for *sea*, *seb*, *seg*, *seh*, *sei* and *sej* genes were performed by which the existence of a 521bp segment was related to the amplification of a specific fragment of gene *sea* that is responsible for enterotoxin type A (Fig.1, lane 9). DNA amplification fragments of 667 bp for staphylococcal enterotoxin are related to B gene (*seb*) (Fig.1, lane 1,5and 8), 287 bp for staphylococcal enterotoxin G gene (*seg*) (Fig.1, lane 1 ,5 and 8), 360bp for staphylococcal enterotoxin H gene (*seh*) (Fig.1, lane 1,4,5) , 454 bp for staphylococcal enterotoxin I gene (*sei*) (Fig.1, lane 2 and 8) and 142 bp to staphylococcal enterotoxin J gene (*sej*) (Fig.1, lanes 1,5 and 8). Also, *S. epidermidis* was used as a negative control and did not yield a PCR product (Fig.1, lane 3 and 7).

A total of 22.5 % of the tested *S. aureus* isolates were positive for one or more toxin genes. 10 percent of total isolates were *sea* positive, 5 % *seb* positive, 17.5 % *seg* positive , 12.5 % *seh* positive, 10 % *sei* positive and finally,10 % *sej* positive.

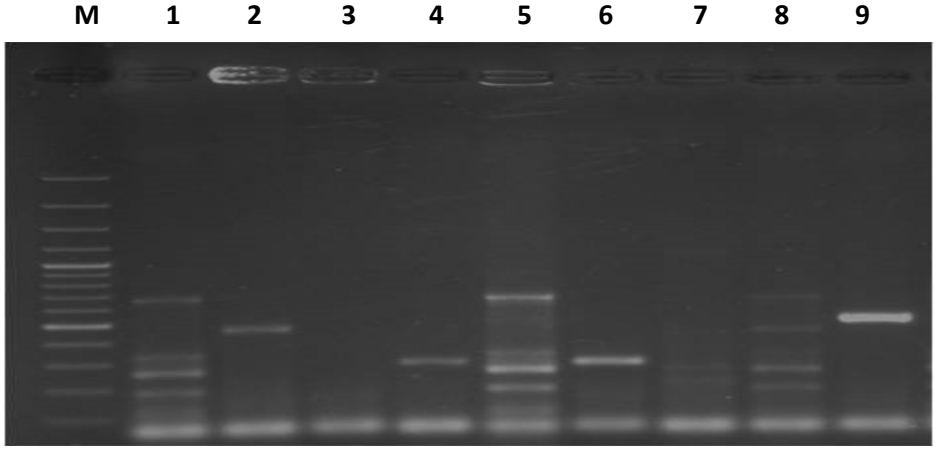

Fig1. Multiplex PCR assays for the detection of enterotoxin genes in *S.aureus* strains.

Lane M, 100 bp DNA marker ; lane 1and 5, *sej* (142 bp), *seg* (287 bp), *seh* (360 bp) and *seb* (667 bp); lane 2, *sei* (454 bp); lane 3 and 7, negative control (*S. epidermidis*); lane 4 and 6, *seh* (360 bp); Lane 8, *sej* (142 bp), *seg* (287 bp), *sei* (454 bp) and *seb* (667 bp); Lane 9, *sea* (521 bp).

DISCUSSION

S. aureus, produces enterotoxins and is one of the common agents of food poisoning [1,24]. This bacterium can persist in anterior nares of healthy human carriers [5]. It is estimated that 30-50% of human population carries *S. aureus* [8]. Accordingly, The human anterior nares and finger tips are important sources of contamination of food products via respiratory secretions or direct contact with hands during manufacturing and handling of food products [8].

In the persent study, we have described a Multiplex PCR-based diagnostic protocol to detect the genes for enterotoxins A, B, G, H, I and J in *S. aureus* isolated from nasal carriers. This technique can identify strains harboring the toxin genes and is independent of the expression and secretion of the toxin [15].

Multiplex PCR results obtained in this study showed that 22.5 % of *S. aureus* isolates were positive for one or more enterotoxin genes. This prevalence rate is in agreement with reports in clinical isolates in Jordan (23%) [17], and in isolates collected from Botswana's (21%] food handlers [13] The prevalence rate is also similar to what was found in nasal isolates in Brazil (22.1%) [24].

But the result of the present study was lower than what was reported in German clinical (43%) and nasal (39.5%) isolates [3], turkey clinical (57.5%) isolates [6], and isolates collected from Japanese (76%) patients with food poisoning [19].

In this study, 10 percent of *S. aureus* isolates were *sea* positive, 5 % *seb* positive, 17.5 % *seg* positive , 12.5 % *seh* positive, 10 % *sei* positive and finally, 10 % *sej* positive.

The predominant enterotoxin type in this study was *seg* (17.5%). Similarly, other studies [3,5, 6,7,18] showed that *seg* gene was dominant in the tested clinical and nasal *S. aureus* isolates.

The *seh* gene was detected in 12.5% of *S. aureus* isolates. It was reported in 15 % of invasive and in 18 % of carrier strains by Peacock et al. [20] , in 6.2 % of strains by Becker et al.[3] and in 8.3% of strains by Collery et al.[5].

The sea gene was present in 10% of *S. aureus* isolates. Less frequently than prevalence of strains isolated from blood and nasal specimens (15.9 %) [3] Described earlier, and nasal specimens from healthy people (20 %) [16]. However, the findings are in agreement with what was reported by the University Hospital in Magdeburg, Germany (10.9 % of MSSA) [12].

The *sei* gene was detected in 10% of *S. aureus* isolates. This prevalence rate is lower than what was reported by Peacock et al. [20], who reported the detection of *sei* in 52 % of invasive strains, and Becker et al. [3], who, similarly, found a prevalence of 55 %.

The *sej* gene was detected in 10% of *S. aureus* isolates. Becker et al. [3] reported the isolation of *S. aureus* with the *sej* gene from blood in 10.5 % and nasal specimens in 3.3%, and Layer et al. [12] in 15 %.

The *seb* gene was detected less frequently: in 5% of *S. aureus* isolates. The results are comparable to the detection of *seb* in 5.9 % of strains from blood [3], but lower than those published by Collery et al. [5] (50 % strain of nasal specimens) and by Peacock et al. [20] (7 % of carrier strains, 9 % of invasive strains).

The variation in reported rate results is probably due to differences in study populations, sampling, and culture techniques. The results may have further been affected by the different ecological sources of the isolated strains (food, humans, and animals) [3, 15, 28]

In conclusion, we developed an easy and rapid Multiplex PCR system showing high specificity capable of detecting 6 SEs genes of *S. aureus* in one set of reactions. This method is simple, sensitive, low cost, relatively rapid and very specific; in addition, it can identify several genes that encode toxin at the same time.

Besides, on the basis of our examinations and available scientific literature, the conclusion reached is that some strains of *S. aureus* isolated from anterior nares of healthy human carriers produce different type of enterotoxins. Therefore, the presence of *S. aureus* in human anterior nares in general, and enterotoxigenic strains, in particular, can be a potential risk for health.

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