

Identification of Staphylococcus Aureus Enterotoxin Genes Using Multiplex PCR

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ABSTRACT

BACKGROUND AND OBJECTIVE: Staphylococcus aureus is one of the most common causes of food poisoning in the world. Various studies indicated that 18-15 percent aureus strains isolated from different sources are able to produce enterotoxin which known as the main factor of poisoning. The aim of this study was to identify enterotoxin genes, such as: (femA, see, sed, sec, seg, seb, sea) in S. aureus confirmed using multiple PCR method.

METHODS: In this study, 60 samples of Staphylococcus aureus isolated from purulent infections, skin and have symptoms of poisoning: vomiting and diarrhea in humans, production of enterotoxin was examined. After DNA extraction of isolates, multiple PCR using specific primers for enterotoxin genes was performed.

FINDINGS: Overall, 50% of Staphylococcus aureus isolates contain one or more enterotoxin gene. The most abundant gene was sea (30%) and sed (10%), see (3.8%), sec (6.1%) were also identified.

CONCLUSION: It was found that other genes such as TSST-1 are involved staphylococcus aureus enterotoxin production in the creation of visual acuity in addition to above genes. In general, the presence of Staphylococcus aureus in human clinical specimens, especially enterotoxigenic strains, can be considered a potential risk for health.

KEY WORDS: *Staphylococcus aureus, enterotoxin gene, multiplex polymerase chain reaction, food poisoning.*

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Introduction

Over the past decade the incidence of microbial foodborne diseases has been increased not only in developing countries with poor hygiene, but also in developed countries with a high standard of health (1). In this regard, numerous reports of illness caused by food contaminated with food pathogens including infection and food bacterial poisoning caused by *Salmonella*, *Staphylococcus aureus*, *shigella*, *listeria monocytogenes*.

Among food bacterial pathogen, *Staphylococcus aureus* is remarkable and significance (2). The report of Casman and colleagues in 1967 indicated that the strength of diversity of *S. aureus* enterotoxin strains isolated from clinical sources about was 47 percent and about 31 percent were non-clinical strains (3). *Staphylococcus* is belong to family of micrococcaceae and is a gram-positive, immovable, non-aerobic, anaerobic spore bacteria.

Four clinically important species: *Staphylococcus aureus* (golden), *Staphylococcus epidermidis*, *staphylococcus aureus saprophyticus* and *staphylococcus lugdunensis* (4).

In this kind of food poisoning, skin wounds, boils and sometimes cause some very dangerous hospital infections and are resistant to treatment (5,6). *Staphylococcal* enterotoxin include types: R, Q, P, O, N, M, L, K, J, I, H, G, E, D, C, B, A, and their associated genes are (ser, seq, sep, seo, sen, sem, sel, sek, sej, sei, seh, seg, see, sed, sec, seb, sea) (7).

Enterotoxin amount needed to make the symptoms of food poisoning is very low. Thus, *staphylococcal* enterotoxin sensitive detection is required even in small amounts.

Nowadays many molecular diagnosis methods such as PCR and multiplex PCR are used to identify *staphylococcal* enterotoxin genes, but the methods of molecular diagnosis, or even in the absence of antigen required to reduce the amount of toxin or toxin genes are detectable (7).

The amount of enterotoxin genes is different based on bacterial origin to the animal, human, infection, food or environment (8,9). In this study, strains of *Staphylococcus aureus* enterotoxin gene encoding the J, I, H, G, and B was performed using multiplex PCR technique.

Molecular characterization of *Staphylococcus aureus* genes (femA, see, sed, sec, seg, seb, sea) isolated from human clinical specimens by multiplex PCR was performed.

Methods

In this study, 60 cases of *staphylococcus aureus* were considered for this study and were kept in the microbiology lab.

Microbial tests: samples in the laboratory with flames under the hood and in accordance with the principles of sterility, on blood agar (Mrk.lman) supplemented with 5% sheep blood were cultured and were incubated for 48 hours at a temperature of 37 °C. For identification of grown bacteria after transferring on nutrient media, the standard microbiological methods including gram staining, catalase and coagulase production tests, growth in mannitol salt agar medium and DNase medium were used (10).

Coagulase test: This test is used for differential diagnosis of *staphylococcus*. Coagulase test done in two ways, slide and tube and pipe method is more accurate. Coagulase attached to the cell wall of *staphylococcus* directly converts fibrinogen into insoluble fibrin and causes the *staphylococcus* to be clot. Free coagulase is done mainly by a tube and connected coagulase test is done on the slide (11).

Fermenting of the mannitol: Although *staphylococcus* ferments various sugars such as sucrose, glucose, ribulose, lactose and other sugar, but fermentation of mannitol by this bacterium has special significance, because only variety of pathogenic *staphylococcus aureus* ferments this sugar. To perform the tests mannitol salt agar medium is required. This environment with high concentrations of salt (5.7%) inhibits growth of most strains of gram-negative and gram-positive except *staphylococcus aureus*. by breaking of peptone in the environment, red colonies with red purple halo are created. Colonies were inoculated on mannitol salt agar and incubated for 48-24 hours in 35 °C. Colonies of *staphylococcus aureus* was yellow and surrounded by a yellow halo.

DNA extraction: a few pure colonies of *Staphylococcus aureus* from nutrient agar medium containing 5 mL of BHI broth medium were incubated for 24 h at 37°C in water bath. Then 1 ml of sterile BHI broth was poured in 1.5 ml microtubes and was centrifuged for 10 minutes in the 3500 g. In the next step the supernatant is completely drained and 800 ul lysis buffer was added and at a temperature of 65 degrees were incubated in water bath for 30 minutes.

After this stage, the vials containing the lysed cells were centrifuged (12000 g) for 5 minutes at a temperature of 4°C and the supernatant phase was transferred in clean and new 1.5 ml micro-tubes and the

same volume of chloroform-isoamyl alcohol was added at a ratio of 1:24. The collection was shaken gently. Then two created phases in previous stage using 12000 g centrifugation for 5 min at 4°C were separated and by removing the upper layer and transfer to 1.5 ml micro-tubes, 5.0 ml RNAase were added to it was placed in a bain-marie for 30 minutes at 37°C.

After 30 minutes, the vials were collected and equal volume of isopropanol was added and incubated for 30 min at -20°C. Then DNA was deposited using centrifugation for 10 minutes in the 14000 g and the supernatant was drain, and micro-tube containing DNA was reversed to be dried at room temperature. Finally, dried DNA was dissolved in 50 ml of deionized water. To evaluate the quality and quantity of extracted DNA, 5 microliters of DNA dissolved in 50 ml of deionized water were electrophoresed using 1% agarose gel and in all the samples genomic DNA with quality and good concentration was observed (12).

Evaluation of extracted DNA: for testing and evaluation of extracted DNA 1% agarose gel was used. Thus, the extracted samples were mixed at a ratio of 6 to 1 with loading buffer and were loaded in the wells of gel. After completion of the electrophoresis, the gel was stained by ethidium bromide and DNA bands in all samples was observed and documented using UV light.

M-PCR test: In this study, to detect the presence of 6 enterotoxin (femA, see, sed, sec, seb, sea) in tested staphylococcus aureus, multiplex PCR technique was

used (table 1). Primers were prepared from Takapozist Company (Thran.Iran). In this study reference strains of *S. aureus* 25923 ATCC, 29213 ATCC, 33591 ATCC were used as control and *Staphylococcus epidermidis* as a negative control.

Preparation of primers: In this technique, six primers for amplification of each of the genes in the genome of *Staphylococcus aureus* enterotoxin companies were scrambling to prepare for work in the laboratory. Primers as lyophilized according to the manufacturer's instructions before using any of the following preparatory steps were taken.

DNA amplification by PCR: Multiplex PCR was done to identify six enterotoxin using six primer pairs (table 1) in a thermocycler device (Eppendorf). For this reaction 5.2 ml PCR buffer (x10), 1 ml of dNTP, 1 ml of salt (MgCl₂), 2.0 mM of each related primer pair, 1 microliter of the Taq DNA Polymerase enzyme and 1 microliter of DNA template were mixed together and the volume 25 ml of water was brought to the final demonized. According to samples, sterile microtubes were placed inside the rock after coding. The code writing is the sample numbering. Then, the PCR test components were added to the microtubes. In order to determine the identity of grown bacteria on nutrient media after transferring, the standard microbiological methods including gram staining, catalase and coagulase production test, growth on mannitol salt agar medium and DNase medium were used.

Table 1. Characteristics of primers used in multiplex PCR

Target gene	Primer	Sequence	PCR Length (bp)
sea	SEA-f	GGTTATCAATGTGCGGGTGG	102 bp
	SEA-r	CGGCACTTTTTTCTCTTCGG	
seb	SEB-f	GTATGGTGGTGTAACTGAGC	164bp
	SEB-r	CCAAATAGTGACGAGTTAGG	
sec	SEC-1	AGATGAAGTAGTTGATGTGTATGG	451bp
	SEC-2	CACACTTTTAGAATCAACCG	
sed	SED-f	CCAATAATAGGAGAAAATAAAAG	278bp
	SED-r	ATTGGTATTTTTTTTCGTTC	
see	SEE-1	AGGTTTTTTCACAGGTCATCC	209bp
	SEE-2	CTTTTTTTTCTTCGGTCAATC	
femA	femA-1	AAAAAAGCACATAACAAGCG	132bp
	femA-2	GATAAAGAAGAAACCAGCAG	

Results

The results of M-PCR test for staphylococcus aureus: At this stage, the search for enterotoxin genes femA, see, sed, sec, seb, sea was performed by using multiplex PCR on all 60 sample, a gene fragment of 102

bp indicates sea gene, a fragment of 164 bp indicates the seb gene, fragment of 451 bp indicates the sec gene, fragment of 278 bp indicates the sed gene, fragment of 209 bp indicates the see gene and fragment of 132 bp

indicates the femA gene that this gene is important in the resistance of *S. aureus* to methicillin. In this study reference strains of *Staphylococcus aureus* 25923 ATCC, 29213 ATCC, 33591 ATCC were used as positive controls and *Staphylococcus epidermidis* was used as a negative control.

M-PCR test results for staphylococcus aureus strains: In this study, 30 samples (50%) from all 60 samples contained one or more of *Staphylococcus aureus* enterotoxin gene. Among the 30 samples, 18 strains (30%) of sea gene, 1 strain (1.6%) containing the sec gene, 6 strains (10%) of sed gene, 5 strains (8.3%) carriers see gene. femA, seb genes were not detected in any of the samples (Fig 1, 2, 3).

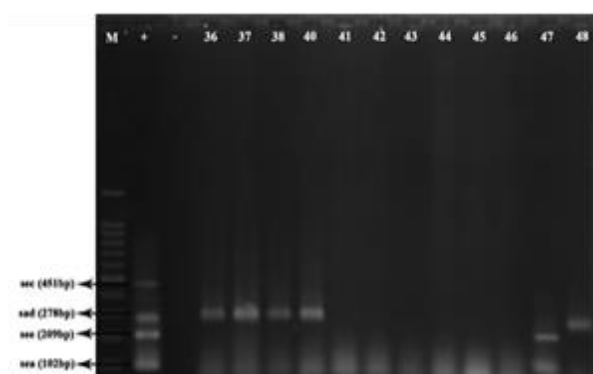


Figure 1. Reaction of M-PCR to detect *Staphylococcus aureus* enterotoxin genes column M: size marker 100 bp, positive control with sea gene (bp102), see gene (209bp), sec gene (451bp), negative control (*Staphylococcus epidermidis*), sample 1, 2, 3, 4, 7, 8, 9, 11, 12 containing sea gene (bp102), samples 2 and 11 with see gene (bp209)

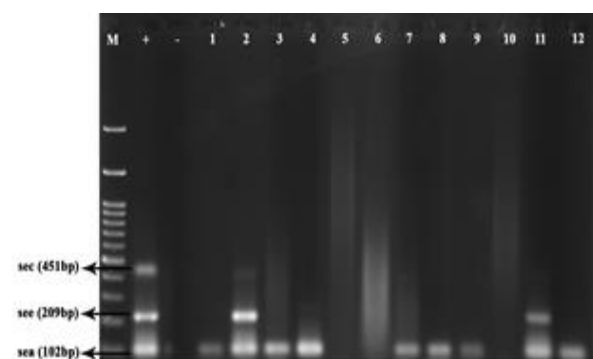


Figure 2. M-PCR reaction for identification of *Staphylococcus aureus* enterotoxin genes. Column M: size marker 100 bp, positive control including sea gene (bp102), see gene (209bp), sec gene (451bp), sed gene (278bp), negative control (*Staphylococcus epidermidis*) sample 47 with see gene, sample 36, 37, 38 and 39 including sed gene

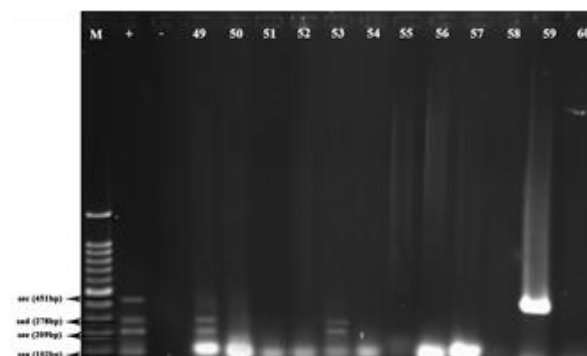


Figure 3. M-PCR reaction for identification of *Staphylococcus aureus* enterotoxin genes. Column M: size marker 100 bp, positive control including sea gene (bp102), see gene (209bp), sec gene (451bp), sed gene (278bp), negative control (*Staphylococcus epidermidis*). Sample 49, 50, 51, 52, 53, 54, 56, 57 including sea gene, sample 49 and 53 with see gene, sample 49 and 53 containing sed gene. Sample 59 with sec gene

Discussion

In this study of 60 samples of *Staphylococcus aureus*, 30 samples (50%) contained at least one enterotoxin gene. abundant gene was sea (30%) and followed by sed (10%), see (8.3%), sec (1.6%) which were less frequently detected.

In the present study, M-PCR techniques was used to identify genes producing enterotoxin, which is a specific, sensitive, rapid and inexpensive method and has the ability to detect multiple genes producing enterotoxin at the same time. The undeniable advantages of this method is identifying potential agents of food poisoning even before the creation of the toxin that by recognition of healthy carrier and the hotspots detected especially in food preparation centers, we can prevent staphylococcal food poisoning.

In humans, the bacteria is anterior side of adults' nasal and in 20 to 30% of the human population is stable and permanent and frequently seen in 60% of cases. Therefore, residents operate in places for preparation, processing and distribution of food are able to transfer the bacteria to food in the event of non-compliance health issues (6,7,9).

In Iran, Iman Fooladi and colleagues, in their study showed that 45% of all isolates of *S. aureus* isolated from skin of 200 patients were producer of enterotoxin (13). In another study Anvari and colleagues showed that from 50 clinical isolates of *Staphylococcus aureus* isolated from wound infections, 74% contained the enterotoxin (14). Barati et al reported that the frequency of sea gene in clinical samples was 6.47% (26). In

another study by Saadati and collaborate on healthy carrier samples using PCR technique, the frequency of sea gene was reported 25.3% (15). Gadyari and colleagues in a study of 100 samples of Staphylococcus aureus isolated from burn patients admitted to hospital, 12% of the samples had sea gene and 1% of samples had seb gene (16). Pinto et al showed that 31% of 131 S. aureus isolates from food containing one or more enterotoxigenic genes. Among them sea gene with a frequency of 17% was reported which is consistent with the results of our study (17).

In the study of Trnikova et al. in Slovakia, 37% of Staphylococcus aureus enterotoxin contamination was reported in ready-to-eat foods, which indicates the ability of Staphylococcus aureus bacteria to grow in a variety of foods (18). In a study by Hwang and colleagues in Korea showed 50% of 143 strains of Staphylococcus aureus isolated from pork and chicken are containing at least one enterotoxin. In this study, the prevalence of sea gene in 7% (10 samples) were reported and seb genes were observed in any of the isolates. The genes seg and sei enterotoxin of Staphylococcus aureus were reported as the most common type with a prevalence of 37% (53 samples) (19). People at risk, such as children, the elderly and the immunocompromised population in our country is remarkable and the issue taking into account the high level of its colonization in healthy individuals up to 60-

50% in the nasopharynx and 5-30% in skin and hair with permanent colonization of 10-20%, particularly by the authorities of prepared foods becomes highlighted (20). In this study, of 60 samples of Staphylococcus aureus, 50% (30 samples) contained one or more enterotoxin gene. This prevalence rate was similar to results reported the frequency of enterotoxigenic strains isolated from nasal Staphylococcus aureus samples in Brazil (22.1%), clinical samples in Jordan (23%) and samples collected from people working in the production and distribution of food in Botswana (21%). In addition, the reported results were consistent with clinical samples (43%) and isolated from the nose (39.5%) in Germany, from clinical samples in Turkey (57.5%) and from samples isolated from patients with food poisoning (76%) in the Japan.

It was found that Staphylococcus aureus enterotoxin production since the creation of visual acuity in addition to these genes, other genes are involved, such as TSST-1. In general, the presence of Staphylococcus aureus in human clinical specimens, especially enterotoxigenic strains, can be considered a potential risk to health.

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