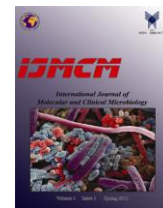




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The prevalence of SEs genes of *Staphylococcus aureus* isolated from patients in Ahwaz hospitals by multiplex PCR

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ABSTRACT

Several virulence factors have been described in pathogenesis of *Staphylococcus aureus* strains. Staphylococcal Enterotoxins (SEs) that is one of the most factors, are belonging to important members of bacterial superantigens. Superantigens are defined by their ability to stimulate cytokine release from both T cells and macrophages. The aim of this study was to evaluate the prevalence of SEs genes of *Staphylococcus aureus* which was isolated from patients in Ahwaz hospitals. This study was carried out only on 231 isolates from clinical samples of patients who were referred to the four hospitals in Ahwaz. *Staphylococcus aureus* isolates were identified by routine bacteriological methods. Genomic DNA was extracted by Phenol-chloroform method. The sets of enterotoxin genes (sea-sei) were amplified by multiplex PCR. PCR products were analyzed by electrophoresis. In this study 231 *Staphylococcus aureus* were isolated from urine, blood, skin chips and spatum samples. 89(39%) strains had enterotoxin genes. The frequency of sea (17%), seg (15%), sec (6%), sed and seh (5.6%), seb (5.1%) and sei (9.5%) were reported. There was no see gene among isolates. The study of genes simultaneously showed that the frequency of two genes and three genes were 20% and 17%. Given the importance of nosocomial *Staphylococcus aureus* infections and the role of SEs as superantigen in the development of various diseases, study of these genes in clinical specimen is necessary.

1. Introduction

Superantigens are defined by their ability to stimulate cytokine release from both T cells and macrophages (Marrack and Kappler, 1990). The proteins bind to relatively invariant regions of major histocompatibility complex II molecules on antigen-presenting cells, and they cross-bridge with certain variable regions of the beta chains of T cell receptors (Vb-TCR) (Kotzin et al., 1993). The alteration in immune system homeostasis occurring in infections caused by superantigens producing bacteria maybe the basis of some clinical manifestation and may be

play an important role in bacterial pathogenesis (Marrack and Kappler, 1990; Scherer et al., 1993; Zumla, 1992). Some bacterial toxins are potent superantigens. Certain strains of *Staphylococcus* or *Streptococcus* produce exotoxins that are major superantigens. Among bacteria, *Staphylococcus aureus* have many important superantigens include Staphylococcal enterotoxins, classically the common causes of food poisoning and nonmenstrual TSS, and TSS toxin 1 (TSST-1), the cause of both menstrual and nonmenstrual TSS and Exfoliative toxins (McCormick et al., 2001). The organism most often colonizes the anterior nares and, from

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there, may colonize other body surfaces, including other mucous membranes and damaged skin. *S. aureus* causes a wide variety of human illnesses, including scalded skin syndrome, toxic shock syndrome (TSS), and necrotizing pneumonia (C.D.C.P., 1999; Daum et al., 2002; Dufour et al., 2002; Kravits et al., 2005; Ladhani, 2001; McCormick et al., 2001; Todd et al., 1978).

Several virulence factors implicated in the pathogenesis of *S. aureus* strains have been described previously (Kuroda et al., 2007; Normanno et al., 2007; Sandel and McKilli, 2004). Staphylococcal Enterotoxins (SEs) is one of the virulence factors that well studied. Staphylococcal enterotoxins (SEs) belong to a large family of staphylococcal and streptococcal pyrogenic exotoxins (PE), sharing common phylogenetic relationships, structure, function, and sequence homology. These toxins cause toxic shock-like syndromes and have been implicated in food poisoning and several allergic and autoimmune diseases (Balaban and Rasooly, 2000). Enterotoxins from *S. aureus* strains can be classified into 18 serological types: A-U (except S, F and T) (Dinges et al., 2000; Holtfreter and Broker, 2005; Letertre et al., 2003; Omoe et al., 2003; Orwin et al., 2003, 2001, 2002).

Staphylococcal enterotoxin serotypes A–E (SEA–SEE) and SEG–SEQ have been well described in the literature. SEA–SEE and SEI are capable of causing vomiting and diarrhea when administered to monkeys and, thus, are correctly referred to as staphylococcal enterotoxins (McCormick et al., 2003). The remaining staphylococcal enterotoxins either lack emetic activity (SEG, SEK, SEL, and SEQ) or have not been tested for emetic activity. According to the suggestions of a recent nomenclature committee, these superantigens are more correctly designated as staphylococcal enterotoxin-like (SEL) (SEL-G, -H, -J, -K, -L, -M, -N, -O, -P, and -Q) (Lina et al., 2004).

Staphylococcal enterotoxin genes are encoding by mobile genetic elements such as bacteriophage (*sea*, *see*, *sep*) and plasmid (*sed*, *sej*, *ser*, *ses*, *set*) or by the pathogenicity islands (SaPIs) on Staphylococcal chromosome castes (*seb*, *sec*, *seh*, *sei*, *sek*, *sel*, *sem*). Therefore these genes are widely horizontally transferred. SEs functions not only as potent gastrointestinal toxins but also as superantigens that stimulate

non-specific T-cell proliferation. Although these are two separate functions localized on separate domains of the proteins, there is a high correlation between these activities and in most cases a loss of superantigen activity (because of a genetic mutation) results in loss of enterotoxin activity as well (Harris et al., 1993). Given the importance of nosocomial *Staphylococcus aureus* infections and the role of Staphylococcal enterotoxin as superantigen in the development of various diseases, the Surveys to detect classical enterotoxins and enterotoxin genes in *S. aureus* from clinical samples have been conducted in some area of Iran. However, there are no published reports about presence of SEs genes in Ahwaz-Iran. Therefore, this study was conducted to investigate the presence of SEs genes of *Staphylococcus aureus* strains isolated from patients in Ahwaz hospitals.

2. Materials and Methods

This descriptive study was performed on 1280 clinical samples from patients who were referred to the four hospitals in Ahwaz (Sina, Abuzar, Golestan and Aria). Samples were collected from urine, blood, skin chips and spatium of patients. After sampling, swabs were immediately transferred into 5 mL nutrient broth and incubated for 18-24 h at 37°C. *Staphylococcus aureus* isolates were identified by by routine bacteriological methods includes colony morphology (gram positive, cluster-forming cocci), positive catalase and coagulase activity, culture on Mannitol salt agar and DNase agar. All identified isolates were stored on BHI broth at -80°C until usage.

2.1. DNA extraction and multiplex PCR amplification

DNA of all *Staphylococcus aureus* isolates were extracted by Phenol-chloroform method, previously described (Vaez et al., 2011). The released DNA was transferred into a new Eppendorf tube and stored at -20°C for used. The primer sets used for enterotoxin genes that were designated by gene runner software are listed in table 1. The amplification of enterotoxin genes were performed by multiplex PCR. Each PCR reaction mix (10 µL) contained 1X PCR buffer, 1 U Taq DNA polymerase, 1.5 mM MgCl₂, 3pmol (3pmol forward & 3pmol

reverse) of one primer (SEs genes) and two primer (SEs genes) and 0.5pmol (0.5pmol forward & 0.5pmol reverse) three primer (16S rRNA gene) (table 2), 0.25 mM dNTPs and 100 ng DNA template. DNA amplification was carried out using the thermal cycler (Mastercycler personal, Eppendorf, Germany) according to the following thermal conditions: initial denaturation for 5 min at 95°C followed by 32 cycles at 95°C for 50s for denaturation, and Annealing at 56°C for 50s and extension at 72°C for 50s. Final extension was carried out at 72°C for 10 minutes. After amplification PCR products were analyzed by electrophoresis on 1.5% agarose gel and Acrylamide 12%.

2.2. Statistical Analysis

A χ^2 test was used to study the correlation between the prevalence of the enterotoxin genes in different groups of *Staphylococcus aureus* isolates. Statistical analysis was done using statistical package, SPSS windows version 15. A value of $P < 0.05$ was considered statistically significant.

3. Results

From 1280 clinical samples, 231 *Staphylococcus aureus* strains based on the results of diagnostic tests were detected. 72.3% of strains were collected from urine culture, 14.3% from blood, 6.5% skin chips and 7% from sputum. Electrophoresis analysis of all Multiplex PCR amplicons and their band size is shown in (Figures 1).

Amplifying of Enterotoxin genes by multiplex PCR showed that only (89) 39% of *Staphylococcus aureus* isolates have enterotoxin gene. The prevalence of enterotoxin genes among *Staphylococcus aureus* isolated from urine, blood, skin chips and sputum samples were 38%, 44%, 29% and 38% (figure 2). There was no association between the frequency of each enterotoxin genes and clinical samples or gender of patients ($P < 0.05$).

The frequency of enterotoxin genes include *sea* (40) 17%, *seg* (35) 15%, *sei* (22) 9.5%, *sec*, *sed* and *seh* (13) 6%, *seb* (12) 5% were reported. There was no *see* gene in all *Staphylococcus* isolates (Figure 3).

In this study, the prevalence of enterotoxin genes were analyzed simultaneously. 60% of isolates only had one enterotoxin gene but the frequency of two genes 20% and three genes 17% among the isolates were reported. In addition, isolates with four genes and five genes also were observed with a frequency of only 1%. (Figure 4). The most frequent variant two combinations were *seg* with *sei* genes (8.5%). The *sea* gene was present in many two combinations. The *sec*, *seg*, *sei* genes combinations had most frequency among three combinations variants (6%) and the *seg* gene was present in all three combinations along with other genes specially with *sei* gene (Table 3) as such as four to six combinations.

4. Discussion

Staphylococcus aureus produces extracellular enterotoxin proteins with low molecular weight that are similar to each other in terms of structure and biological activity but they are different as far as antigen characteristics are concerned. These toxins belongs to a large pyrogenic toxins family including classical SEs (SEA through SEE) newly (SEG, SEH, SEI, SEJ, SER, SES, SET) and SE like toxins (SeIK, SeIL, SeIM, SeIN, SeIO, SeIP, SeIQ, SeIU) (Atanassova et al., 2001; Argudín et al., 2010; Thomas et al., 2006). The pyrogenic toxins like *Staphylococcal* TSST-1 and enterotoxins act as superantigens that have ability to stimulate large populations of T cells (~20–30%) leading to the production of a cytokine bolus (Balaban and Rasooly, 2000; Choi et al., 1989). Our results showed that only 39% of *Staphylococcus aureus* clinical isolates had enterotoxin genes but results from different reports have shown the high incidence of enterotoxin genes among food-borne *Staphylococcus aureus* isolates (Argudín et al., 2010). Our results are relatively similar to Fooladi et al in 2007 study that the prevalence of SEs genes in clinical sample 45% have been reported (Imanifooladi et al., 2007). Nowroozi et al in 2012 showed that the prevalence of SEs genes was 57.14% in clinical samples (Nowroozi et al., 2012). The frequency of SEs genes among *S. aureus* isolates in our region was lower than other studies performed in various regions. In a study by Kamarehei in Gorgan, among the 170 isolates of *S. aureus*, 60.6% and 27.1% contained *sea* and *seb* genes,

respectively that was higher than our study (Kamarehei et al., 2013). This variation may be related to the different epidemiological factors such as region, type of strain, type of specimen and the patients.

Klots et al in a study in 2003 have been reported the prevalence of SEs genes 47.31% in clinical samples (Klotz et al., 2003). In a study by Adwan et al in 2013 the prevalence of SEs genes 33% have been reported (Adwan et al., 2013). The most common Staphylococcal enterotoxins are SEA and SEB (Klotz et al., 2003). SEA is the most common toxin in *Staphylococcus*-related food poisoning. SEB, while it is associated with food poisoning, has been studied for potential use as an inhaled bioweapon (Ler et al., 2006).

Among the genes examined, the frequency of *sea* gene 17%, *seb* 5%, *sec* 6%, *sed* 6.5%, *seg* 15%, *seh* 6.5% and *sei* gene 9.5% were reported. Our results are relatively similar to a study by Nowroozi et al in 2012 that the prevalence of *sea* gene 20% and *sed* gene 5% have been reported (Nowroozi et al., 2012). Saboui et al showed that in Iranian patients in Referral Children's Hospital the prevalence of *sec* gene is 3% and *sed* gene is 4.5% (Sabouni et al., 2014). In a study by Ataee in 2011 the prevalence of *seb* gene 5% have been reported (Ataee et al., 2011). Ran Peck et al reported the prevalence of *sea* gene 21.4%, *seb* gene 5.8%, *sed* gene 2.9% and *seh* gene 7.2% in blood samples of Korean patients and in nasal clinical sample *sec* gene 2.1% has been reported (Peck et al., 2008). In a study by Adwan et al in 2013 in Palestine the prevalence of *seg* gene 22.2%, *seh* gene 12.3%, *sei* gene 8.6% has been reported (Adwan et al., 2013) and in Thailand, one study reported that the prevalence of *sec* and *sed* were 7% and 5% that are similar to our results (Indrawattana et al., 2013). SEG, SEH, and SEI are not as well studied as the others, but were associated with one of the food poisoning outbreaks in Taiwan (Chen et al., 2004). SEH has been also identified as one of the causes of massive food poisoning associated with the reconstituted milk consumption in Osaka, Japan in 2000 (Ikeda et al., 2005). Previous studies demonstrated that SEA, SED, and SEE proteins share 70–90% sequence homology, while only 40–60% with SEB, SEC (Al-Daccak et al., 1998; Balaban and Rasooly, 2000). In this study there was no *see* gene in all isolates. Our results are relatively

similar to Ran Peck study (Peck et al., 2008). Previous study have been shown that SEE has also been documented in some cases of food poisoning (Morris et al., 1972). Our results showed that the prevalence of *seg* and *sei* genes were 15% and 9.5%. It was shown that new enterotoxin genes mainly *seg*, *sei*, *sek* and *seq* were found in isolates from patients rather than strains from food samples and food poisoning (Zhang et al., 2011). Also clinical *Staphylococcus aureus* strains involved in staphylococcal toxic shock syndrome and scarlet fever without producing classical SE's or toxic shock syndrome toxin 1, leading to the suggestion that SEG or SEI toxins caused these diseases (Jarraud et al., 1999). The Products of *seg*, *seh* and *sei* genes showed emetic activity with animal model (Harris et al., 1993), but it is not yet clear if *seg* and *sei* genes are responsible for food poisoning in humans. Among the isolates some of them (20%) had two genes combination and some had three genes combinations (17%). The most frequent variant two combinations were *seg* with *sei* genes (8.5%) and the *sea* gene was present in many two combinations. Also the *sec*, *seg*, *sei* genes combinations had most frequency among three combinations variants (6%). The coexistence of the *seg* and *sei* genes might be due to their chromosome location as both genes are found in atandem orientation and belong to an operon, called the enterotoxin gene cluster (*egc*), which contains five enterotoxin genes (*seg*, *sei*, *sem*, *sen* and *seo*) and two pseudogenes (*u ent1* and *u ent2*) (Jarraud et al., 2001). In a study conducted in 2011 Vasconcelos et al showed a combination gene *seg* + *sei* that had toxigenic potential in strains isolated from patients with CNS (Coagulase-Negative Staphylococci) (Vasconcelos et al., 2011). In a study Ran peck et al showed high frequency of combination gene *seg* + *sei* in Korean patients (Peck et al., 2008). The results showed that *seg* and *sei* genes almost are more substances.

Conclusion

Given the importance of nosocomial *Staphylococcus aureus* infections and the role of SEs as superantigen in the development of various diseases, study of these genes in clinical specimen is necessary.

Table 1. The nucleotide sequences of primers used for multiplex PCR.

primer	Nucleotide sequences	Size (bp)
16S rRNA	F:5'-GTA GGT GGC AAG CGT TAT CC-3'	228 bp
	R: 5'- CGC ACA TCA GCG TCA G-3'	
sea	F:5'- TTC ATT GCC CTA ACG TTG AC-3'	622 bp
	R: 5'- CCG AAG GTT CTG TAG AAG TAT G-3'	
seb	F:5'- CCT AAA CCA GAT GAG TTG CAC-3'	491 bp
	R: 5'- CAC CAA ATA GTG ACG AGT TAG G-3'	
sec	F:5'- TTT TAT GTC TAG TTC TTG AGC TG-3'	367 bp
	R: 5'-GTC TGT AGA TAA ATT TTT GGC AC-3'	
sed	F:5'- GTG GTG AAA TAG ATA GGA CTG C-3'	385 bp
	R:5'- CAA TAT GAA GGT GCT CTG TGG-3'	
see	F:5'- ATG GTT TAA GCA ATC TTA GGC-3'	531 bp
	R: 5'- GTC TAT CGA CAA GTT AAT TGG TAC-3'	
seg	F:5'- TGA ATC TTT ATA CGT CTC CAC C-3'	326 bp
	R: 5'- ATT GTC TAT TGT CGA TTG TTA CC-3'	
seh	F:5'- CTG TTT GAA TAC CAT CTA CCC-3'	270bp
	R:5'- TGA AGA GTG ATG AAA TAA GTG G-3'	
sei	F: 5'- CAA CTC GAA TTT TCA ACA GGT AC-3'	466bp
	R:5'- TAC AGC CAG TCC ATC TCC TG-3'	

Table 2. The primers used in the PCR reaction.

	Mix 1		Mix2		Mix3		Mix4	
	Name primer	PCR product	Name primer	PCR product	Name primer	PCR product	Name primer	PCR product
Three primer	16 S rRNA	228bp	16 S rRNA	228bp	16 S rRNA	228bp	16 S rRNA	228 bp
Two primer	Seg	326bp	Sed	385bp	Seb	491bp	Sea	622 bp
One primer	Sei	466bp	See	531bp	She	270bp	Sec	367bp

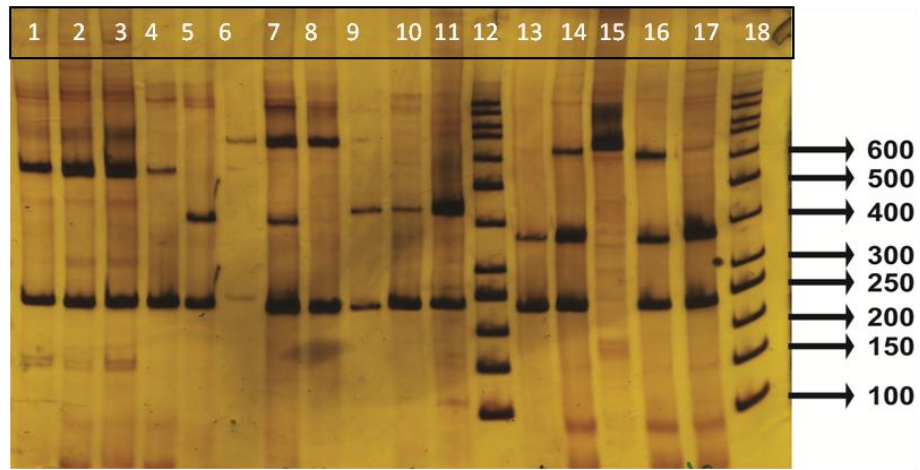


Figure1. Acrylamid gel electrophoresis analysis of multiplex PCR products of enterotoxin genes. Lane 1,2,3,4, *seb* gene(491 bp), Lane 5,7 *sed* gene(385 bp), Lane 6 *seh* gene(270 bp), Lane 6,7,8 ,14-17 *sea* gene(622 bp), Lane 9-11 *sei* gene(466 bp), Lane 13,14,16,17 *sec* gene(367 bp), Lane12, 18 100 bp Ladder, All 228 bp size bands are *16S rRNA* gene

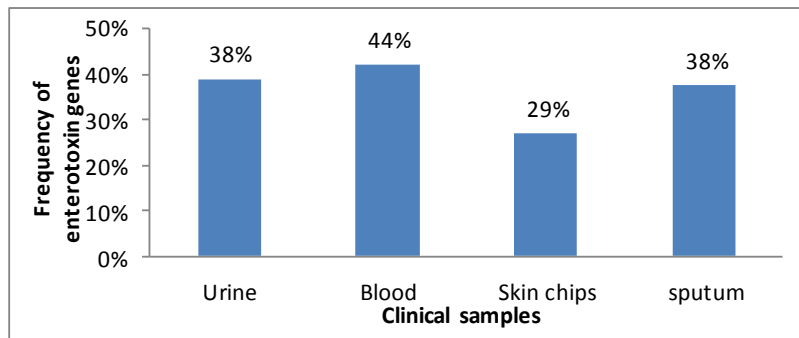


Figure2. The frequency of enterotoxin genes in *Staphylococcus aureus* strains isolated from clinical samples.

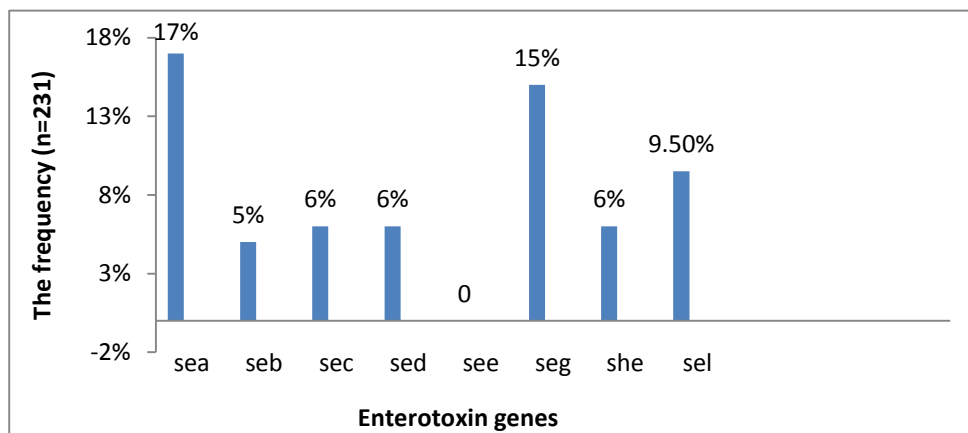


Figure3. The frequency of enterotoxin genes in *Staphylococcus aureus* isolates

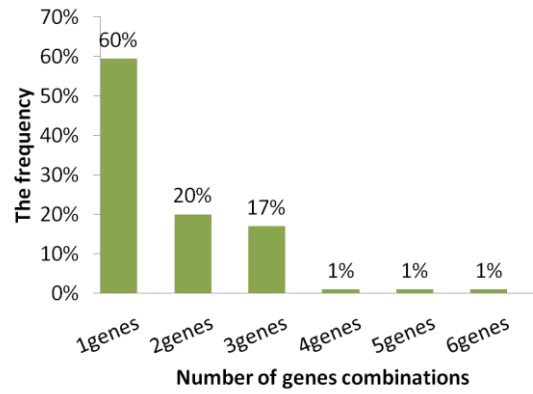


Figure 4. The frequency of genes combinations among *Staphylococcus aureus* isolates (n=89)

Table 3. The frequency of enterotoxin genes combination

Enterotoxin gene combinations	n (%)
<i>Sea</i>	24 (27%)
<i>Seb</i>	6 (7%)
<i>Sec</i>	4 (5.4%)
<i>Sed</i>	4 (5.4%)
<i>Seg</i>	8 (9%)
<i>Seh</i>	7 (8%)
<i>sea+seb</i>	3 (3%)
<i>sea+sec</i>	3 (3%)
<i>sea+sed</i>	1 (1%)
<i>sea+seg</i>	2 (2.5%)
<i>sea+she</i>	1 (1%)
<i>seb+she</i>	1 (1%)
<i>seg+sei</i>	7 (8.5%)
<i>sea+sed+seg</i>	1 (1%)
<i>sea+seg+sei</i>	1 (1%)
<i>sea+seg+she</i>	1 (1%)
<i>seb+seg+she</i>	1 (1%)
<i>sec+seg+sei</i>	5 (6%)
<i>sed+seg+sei</i>	4 (4.5%)
<i>seg+seh+sei</i>	2 (2.5%)
<i>sea+sed+seg+sei</i>	1 (1%)
<i>sea+sec+sed+seg+sei</i>	1 (1%)
<i>sea+seb+sec+sed+seg+sei</i>	1 (1%)

Refereces

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