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Prevalence of enterotoxins B and C in clinical isolates of *Staphylococcus aureus* from Southwest of Iran

Sajad Ababaf[1], Abdolmajid Ghasemian[1], Hossein Motamedi[2], Farshad Nojoomi[1*]

[1]Microbiology Department, Faculty of Medicine, AJA University of Medical Sciences, Tehran, Iran
[2]Biology department, Faculty of science, Shahid Chamran University of Ahvaz, Ahvaz, Iran

***Correspondence to**
Farshad Nojoomi, Email: fnojoomi2@gmail.com

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Abstract

Introduction: *Staphylococcus aureus* enterotoxins play an important role in the incidence of food poisoning and various other syndromes are occurred such as staphylococcal scalded skin syndrome and toxic shock syndrome.
Objectives: The purpose of the present study was to investigate the frequency of enterotoxin B and C genes among *S. aureus* isolates from clinical samples obtained from inpatients of a hospital by polymerase chain reaction (PCR).
Materials and Methods: This cross-sectional study conducted during the period July to December 2015. Clinical samples including blood, urine, wounds and nasal mucosa samples were collected from a hospital in Ahvaz, southwest of Iran and screened for *S. aureus* strains by several phenotypic and biochemical tests. A total of 132 clinical samples were collected from which 60 samples were infected with the *S. aureus* strains. Then PCR assay was carried out to determine the *entB* and *entC* genes prevalence among these isolates.
Results: Molecular analysis revealed that only 8 (13.3%) isolates harbored the *entB* and/or *entC* genes. Five strains (8.3%) contained only *entB* gene, 2 strains (3.3%) contained *entC* gene and only one strain (1.7%) was simultaneously positive for both *entB* and *entC* genes. There was no significant difference among various age groups regarding enterotoxin genes ($P=0.551$).
Conclusion: These results demonstrated that enterotoxin-producing strains have a relatively low incidence in clinical samples in understudy hospital, but monitoring of their prevalence is necessary in regular screening programs in order to find the possible increase in their prevalence and prevention of their outcomes.

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Introduction

Staphylococcus aureus strains play an important role in the incidence of clinical infections and food poisoning, as far as these microorganisms are introduced to the second or third cause of food poisoning (1). Virulence factors of *S. aureus* are exotoxins, surface proteins, extracellular enzymes and polysaccharide capsule (2). On the other hand, one of the most important pathogenic factors of this bacterium is enterotoxins family (3). Staphylococcal enterotoxins (SEs) are proteins with 26-29 kDa molecular weights that are heat-stable and resistant to pepsin digestion and thus can cause gastrointestinal disease and food poisoning (4). SEs and toxic shock syndrome toxin-1 (TSST-1) are super-antigens that mean they can activate a large population of T helper lymphocytes (CD_4^+) leading remarkable cytokine secretion, immune system disorders and even death (5). Each of SEs do this by non-specific binding to a portion of class II MHC (major histocompatibility complex), for example staphylococcal enterotoxin B (SEB) bind to the alpha chain whereas enterotoxin E (SEE) bind to the beta chain of the class II MHC molecule (6,7). Today molecular methods, including polymerase chain reaction (PCR) and multiplex PCR as well as immunological techniques are used to identify enterotoxins-encoding *S. aureus* (8,9). Usually, enterotoxins-encoding genes are located on transposable genetic elements such as plasmids, prophages or

Key point

To investigate the frequency of enterotoxin B and C genes among S. aureus isolates from clinical samples obtained from in-patients of a hospital, we found that enterotoxin-producing strains have a relatively low incidence in clinical samples in a hospital of Ahvaz. However, monitoring of their prevalence is necessary in regular screening programs in order to find the possible increase in their prevalence and prevention of their outcomes.

pathogenicity islands (10). Totally, enterotoxins family is consisted of 20 members that based on their biological function and serological characteristics are classified into two subfamilies of classic enterotoxins (staphylococcal enterotoxins A and E [SEA–SEE]) and new enterotoxins (staphylococcal enterotoxins G-U [SEG–SEU]) (11,12). It has been proved by many studies that not all of these enterotoxins cause food poisoning. SEA mostly causes *S. aureus* related-food poisoning compared to other enterotoxins (13). SEB while is related to food poisoning, is known as a bioterrorism agent (14). Additionally, SEC that is the third discovered enterotoxin, usually encoded by invasive *S. aureus* isolates, especially methicillin resistant *S. aureus* (MRSA) and is one of the main pollutants in milk that enable causes economic losses in the dairy industry (15,16).

Objectives

The aim of the present study is to investigate the frequency of enterotoxin B and C-encoding *S. aureus* isolated from clinical samples by PCR.

Materials and Methods

Clinical samples including blood, urine, wounds and nasal mucosa samples (5 mL for liquids and a sterile swab for wound and nasal mucosa) were collected during July to December 2015 from a hospital in Ahvaz, southwest of Iran. These samples were obtained from all patients referred to the pathology laboratory of the mentioned hospital. The bacterial isolates were identified based on several phenotypic and biochemical tests, including gram staining, catalase and coagulase analysis, growth on Mannitol salt agar (MSA) medium and susceptibility to furazolidone and resistance to bacitracin.

In order to screening for the presence of *ent*B and *ent*C genes, PCR assay was performed. DNA was extracted by boiling a single colony of *S. aureus* was dissolved in 200 µL milli-Q water and heated at 100˚C for 15 minutes. Then the sample was centrifuged at 5000 rpm for 1 minute and the supernatant (containing DNA) was transferred to another Eppendorf tube. Cold ethanol 99% (Merck, Germany) was added to this supernatant (2.5 V/V) and stored at -22˚C for 1 hour. Following centrifugation at 13000 rpm for 10 minutes the supernatant was discarded and the sediment as template DNA was dissolved in 50 µL milli-Q water and stored at -22˚C.

PCR reaction was carried out by specific primers for *entB and entC* genes (Table 1) in the 25 µL reaction mixture contained 12.5 µL of 2x PCR Master Mix Red (Ampliqon, Denmark), 10 ρmol of each primer, 1 µL of template DNA and 10 µL of milli-Q water. Concentration of $MgCl_2$ was 1.5 mM. The oligonucleotide primers for amplification *entB and entC* genes were selected based on the study of Mehrotra et al (17). PCR reaction was carried out by an initial denaturation step at 94°C for 5 minutes, 35 cycles each consisting of denaturation at 94°C for 2 min, annealing at 58°C for 2 minutes, and extension at 72°C for 1 minute followed by a final extension step at 72°C for 7 minutes in a thermal cycler (Bio-Rad USA). The amplified product was confirmed by electrophoresis in 1.5% agarose gel for 45 minutes at 80 V and documented by UVI gel doc system.

Ethical issues

The research followed the tenets of the Declaration of Helsinki. Consent for the study had been taken. The ethical committee of AJA University of Medical Sciences, Faculty of Medicine approved the research.

Statistical analysis

The obtained data were analyzed by SPSS software (version 22) through chi-square method with 95% confidence interval ($P<0.05$). The *t* test and ANOVA tests were performed considering 95% CI and *P* value <0.05 as a significant result.

Results

In this research, 132 clinical samples were obtained from the pathology laboratory of a hospital that among them 60 (40.5%) *S. aureus* strains were detected after phenotypic and biochemical analysis.

These samples were from blood (16, 26.7%), urine (22, 36.7%), wounds (8, 13.3%) and nasal mucosa (14, 23.3%). From these samples, 78.3% were from men and 21.7% were from women. Also maximum *S. aureus* infection was in the age range of 21 to 30 years (Table 2).

The results of PCR assay indicated that 8 (13.3%) of isolates were harbored with *entB* and/ or *entC* genes among them 5 strains (8.3%) contained only *entB* gene, 2 strains (3.3%) contained only *entC* gene and only one strain (1.7%) were positive for both *entB* and *entC* genes. As depicted in Figure 1, strains of related by column 5 and 6 containing *entB*. From the total of 8 *entB* and *entC*-encoding *S. aureus* strains, maximum frequency belonged to urine by 4 strains (50%). Also, their number in men was more than women (5 to 3 strains). Related data by 8 *entB* and *entC*-encoding *S. aureus* strains are summarized in Table 3.

Table 1. Nucleotide sequences, locations in genes and sizes of PCR products for *entB & entC* genes-specific primers

Gene	Primer	Oligonucleotide sequence (5′-3′)	Location within gene	Size of amplified product (bp)
entB	GSEBR-1	GTATGGTGGTGTAACTGAGC	666–685	164
	GSEBR-2	CCAAATAGTGACGAGTTAGG	810–829	
entC	GSECR-1	AGATGAAGTAGTTGATGTGTATGG	432–455	451
	GSECR-2	CACACTTTTAGAATCAACCG	863–882	

Table 2. Number and frequency (%) of clinical samples infected to *Staphylococcus aureus* based on age range

Age range	Number	%
20 years and under	14	23.3
21-30 years	19	31.7
31-40 years	11	18.3
41-50 years	9	15.0
Over 50 years	7	11.7
Total	60	100

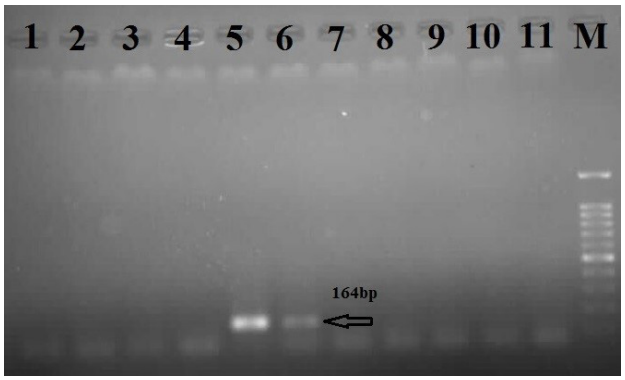

Figure 1. Electrophoresis patterns showing PCR amplification products for the Staphylococcal *entB* and *entC*.
Lane M: DNA Ladder (100 bp). Lanes 1-4 and 7-11: negative sample (*S. aureus* with no *entB* & *entC* genes). Lanes 5-6: related to *entB*-encoding *S. aureus*.

Discussion

As SEs play a major role in pathogenesis of this agent and determining of the prevalence of *S. aureus* strains encoding these toxins is of great importance in order to control of its food poisoning. Identification of these strains is based on both molecular and immunological methods (18,19). Advantage of molecular methods than immunological methods (such as ELISA and radioimmunoassay) is that in the first case, enterotoxin genes expression is not necessary and strains that produce toxin even at low levels can be easily detected (20,21). Thus, we used PCR assay for this purpose.

Staphylococcus aureus strains are widespread in hospitals and can be easily found on the surfaces and also internal space and can make a wide range of hospital infections including hospital acquired methicillin resistant *S. aureus* (HA-MRSA) infections in patients (22). Therefore, determination of *S. aureus* strains prevalence in a hospital can be considered as an indicator for monitoring hospital health and hygiene status. High incidence of this agent in hospital environment indicates low healthcare efficiency.

Enterotoxin B and C were selected in our study, because they can be categorized in a subgroup of enterotoxins. In fact, SEB and the three isotypes of SEC (SEC1, SEC2 and SEC3) have nearly 70% identical at primary structure of proteins (amino acid sequence) leading to similar folding pattern and three-dimensional structure, eventually (23,24).

On the other hand, enterotoxin B was chosen because of its importance in bioterrorism phenomenon as a bioweapon. Hence, this important macromolecule belongs to category B of bioterrorism agents and can easily through contaminated food causes food poisoning (25,26). This toxin is rarely causing death and only high consumption can be fatal. However, high frequency of enterotoxin B-encoding *S. aureus* in a statistical society can be an alarm for a bioterrorism attack, especially in the Middle East that is important politically.

As previously mentioned, the majority of *S. aureus* isolates in dairy products encodes high level of SEC (16). Therefore by high presence SEC in a clinical sample, it seems that people have used the contaminated milk and this shows the importance of studying the enterotoxin C.

The results of current study revealed that from 60 detected *S. aureus* strains just 8 strains (13.3%) possessed enterotoxin B and C -producing genes. In addition, the prevalence of *entB* and *entC* is usually low in other research on enterotoxin genes frequency, while *entA* has usually high prevalence in among all the enterotoxins (27). Previously, Oliveira et al investigated enterotoxin production and enterotoxin gene distribution. Their results showed among the 83 *S. aureus* isolated just 7 (8.4%) isolates possess *entC* as well as *entB* genes was not identified (28).

Similar to our study, in 2015 a research was carried out on the prevalence of enterotoxin genes *S. aureus* isolated from animal originated foods that from 98 *S. aureus* isolates examined, 4.1% and 5.1% carried *entB* and *entC*, respectively and as previously noted, *entA* was the most frequent (11).

Also in an Indian study in 2000, both *entB* and *entC* had

Table 3. Clinical sample type, age range and gender of related by each of 8 *entB* and *entC*-encoding *Staphylococcus aureus* strains

Positive sample	Number	Clinical samples type	Age range	Gender
*ent*B	5	Urine	Over 50 years	Man
		Urine	20 years and under	Man
		Urine	31-40 years	Woman
		Nasal mucosa	20 years and under	Man
		Blood	Over 50 years	Woman
*ent*C	2	Urine	Over 50 years	Woman
		Nasal mucosa	20 years and under	Man
*ent*B & *ent*C	1	Wounds	41-50 years	Man

low frequency (5.6% and 7.5% respectively) (17) that confirms findings of present research.

In other study in 2011, detection of enterotoxin A, B, C and Q genes was carried out, which in line with our findings, *entB* and *entC* had nearly low prevalence by 15.8% and 9.5% respectively (29). In this research the abundance of *entB* is less than the *entC* that confirms our findings.

Like many studies, current study shows that some isolated *S. aureus* strains have more than one enterotoxin gene (30). Also, using statistical analysis we have examined the relationship between *entB* and *entC*-encoding *S. aureus* frequency and clinical samples type, the patient's age and patient's gender. Due to being *P* value ≥ 0.05 in all three statistical analysis, thus no correlation detected between the enterotoxin-encoding strains frequency and the three factors mentioned.

Conclusion

These results demonstrated that enterotoxin-producing strains have a relatively low incidence in clinical samples in one hospital of Ahvaz, but monitoring of their prevalence is necessary in regular screening programs in order to find the possible increase in their prevalence and prevention of their outcomes.

Limitations of the study

This study is a single center study, which further investigations by larger samples are needed.

Authors' contribution

Study concept and design; FN. Acquisition of data; HM. Laboratory work and analysis and interpretation of data; SA and AG.

Conflicts of interest

There is no conflict of interest regarding the publication of this paper.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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