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Molecular Screening of Staphylococcal Enterotoxin Type P Encoding Gene From Clinical Isolates

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Background: In recent years, the roles of Staphylococcal enterotoxins in the non-gastrointestinal diseases have been reported. The most frequently mentioned was enterotoxin type A. But in many cases there are also high similarity with type P. Accordingly, the differentiation of producing enterotoxin type P strains from type A is essential.
Objectives: The objective of this study was to assess and characterize Staphylococcus aureus containing entP gene from infectious specimens.
Materials and Methods: Based on the reference sequence (*S. aureus* N315 entP gene), pair primers were designed. 350 clinical strains of *S. aureus* were assessed by polymerase chain reaction (PCR). The purified PCR product was sequenced. All isolated *S. aureus* strains containing the entP gene were tested by Enzyme immunoassay.
Results: The PCR amplification method was optimized for entP gene detection. The used primer pairs were amplified for 213 bp and 700 bp fragment separately. The sequencing results indicate that only 98 (28%) out of the 350 strains of *S. aureus* contained entP gene. The results of Enzyme immunoassay test for enterotoxins detection revealed that 79 (22.57%) of the strains contained entP gene were which also produced other enterotoxins (such as enterotoxin A to E) and 19 (5.43%) of the strains were carriers of only enterotoxin P gene unable to produce other enterotoxins.
Conclusions: The results revealed, the specific primers that amplified the entE gene were able to amplify the Staphylococcal Enterotoxin-Like Toxin Type P gene. The specific primers for the entP gene were amplified a fragmented gene (700 bp) showed 100% homology with entP reference gene and also 80% homology with entA and entE genes.

Keywords: Staphylococcus aureus; Enterotoxins; Polymerase Chain Reaction

1. Background

The most virulent factors of *Staphylococcus aureus* are enterotoxins (SEs) that cause food poisoning and toxic shock syndrome (1, 2). SEs are potent inducers of cytotoxic T-cell activity and cytokine production in vivo (3). In addition, SEs are being increasingly recognized for their possible roles in many other human diseases, such as atopic dermatitis (4), Kawasaki syndrome (5), nasal polyposis (6) and certain autoimmune disorders (7). Accordingly, to clarify the role of the causative agents of human diseases caused by staphylococcal enterotoxins, it is necessary to have further assays for them (8). However, in terms of frequency and/or seriousness of disease some kind of bacterial toxins play a more important role.

S. aureus is a facultative aerobic Gram positive cocci, colonizes up to 50% of humans (9) and may release enterotoxins. The organism most often colonizes the anterior Nose and may also colonize other parts of body surfaces, including mucous membranes and damaged skin. Staphylococcal enterotoxin serotypes A–E (SEA–SEE) and SEG–SEQ have been widely studied (10, 11). SEA–SEE and SEI are found to be capable of causing vomiting and diarrhea when administered to animals likes monkeys (12). The rest of enterotoxins (SEG, K, L and Q) as superantigens may also have emetic activity or have not been tested for this mode of action (13). Based on current research,

Implication for health policy/practice/research/medical education:
Patients undergoing percutaneous coronary intervention (PCI) are amongst the individuals with highest risk for developing adverse cardiac events and higher morbidity and mortality. There are different reports in various studies about the rates of dyslipidemia control. Present study concluded that lipid-lowering agents and regular follow-up visits did assist in achieving lipid-control goals in our patients, but a large proportion of the patients remained dyslipidemic at the end of the follow-up period. More attention should, therefore, be paid to life style modification, using statins with higher doses, and utilizing other classes of lipid-lowering agents such as ezetimibe or fibrates, if needed, in order to achieve the optimal targets of LDL-C, HDL-C, and TG.

the staphylococcal enterotoxin A (SEA) is the most commonly reported in clinical isolates, especially from food poisoning (11, 14), and it is an extremely potent gastrointestinal toxin; as little as 100 ng of this toxin is sufficient to cause symptoms of intoxication (1). However, the role of this toxin in organs other than gastrointestinal tract remains unclear.

There are several reports that investigated the biological properties of a novel staphylococcal enterotoxin-like toxin type P (SElP) (8, 15). SElP induces a substantial proliferative response and the production of cytokines interleukin-2, gamma interferon, tumor necrosis factor alpha and interleukin-4 from human T cells when administered at a concentration of 0.4 pm (0.01 ng/ml) or more and in mice implanted with an osmotic pump filled with SE-like type P showed 78.1% homologous with staphylococcal enterotoxin type A (16). The results of some reports has showed that the coagulase-negative staphylococci (CNS), isolated from animal and human cases are carrier of genes encoding enterotoxins and enterotoxin-like toxins protein (17, 18).

2. Objectives

The aim of this study was to isolate and characterize the *S. aureus* producer of enterotoxin P and related enterotoxins like proteins from infectious specimens.

3. Materials and Methods

3.1. Criteria for Selection of Bacterial Strains

All patients were examined by a specialist in the infectious disease department and they were then referred to the molecular bacteriological laboratory. The Gram stain, catalase, coagulase, DNase, ability to grow on mannitol salt agar (Lot 105404 752 Merck Germany) were enrolled in this study. The 350 strains of *S. aureus* were isolated from superficial infected tissue; blood, synovial fluid, feces, throat, urine and spinal fluid. The collected isolated strains were maintained at -20°C within 20% glycerol.

3.2. Bacteria Culture and DNA Extraction

Bacterial genomic material was extracted with modified salting out methods (19). To summerize, each single-colony of bacteria was inoculated into LB broth (Lot VM1322685, Merck Germany) medium separately. After 24 hours incubation, 1ml of content was transferred into a 2 ml sterile micro tube and centrifuged (10 min in 5000 × g at 4°C). The supernatant was discarded, and then 400 µl STE buffer (Tris-HCl 10mM, NaCl 10 mM, EDTA 1 mM, pH =7.5) was added to re-suspend the cell-sediment completely. In addition, 125 µl of 2% sodium dodecyl sulfate (SDS) and 250 µl of 3M sodium acetate was added to the tube and the content mixed by gently inverting for 10 times. Afterwards, the tube was centrifuged (5 min in 3000 × g at 4 °C) to lead DNA into supernatant. The supernatant was transferred into a new sterile tube. 750µl of cold absolute ethanol was added and it was kept overnight at -20 °C.

Next day, the tubes were centrifuged (12000 × g for 20 min at 4 °C), the supernatant was discarded and the sediments let to dry. Volume of 750 µl of 70% ethanol was added to sediments and after 1 hour the product was centrifuged (12000 × g for 20 min at 4 °C). The supernatant was discarded again and sediments let to dry completely at room temperature. Then adding 50 µl D.W to each tube and incubating them at 37 °C for 45 min. Applying Nano- Drap (Thermo Scientific NanoDrop 2000 Spectophotometer USA), the concentration of DNA have been measured. 1 µl of genomic product solution transferred into new tubes and stored in -20 °C to use as template for PCR when needed.

3.3. Primer Design

Based on the reference sequence (*S. aureus* N315 enterotoxin P gene; accession number BA000018.3) the pair primers were designed by AllelID V2 software, analyzed by primer3 software and multiple alignments was carried out by DNASIS MAX Trial version. Then, it was synthesis by Cinnagene Company (Tehran, I.R. of Iran).

3.4. DNA Amplification and Sequence Analysis

For DNA amplification, the master mix was made in 200 µl microtubes. By using a 25 µl reaction mixture containing the 5 ng/1µl DNA template, 2 unit of Taq DNA polymerase, 5 µl of 10X PCR buffer II containing 2 mM of each dNTPs, 2 mM MgCl2 (All reagents were from Fermantase, USA), 10 µM of the primer pair (synthesized by Cinnagene Co, I.R. IRAN) and double-distilled water to the final volume of 25 µl. All the amplifications were carried out in a thermal cycler (Bio- Rad, C1000, USA) with initial denaturation at 94 °C for 5 min followed by 32 cycles of denaturation at 94 °C for 1 min, primer annealing at 56°C for 1 min and extension at 68 °C for 1 min, followed by a final extension at 68 °C for 7 min.

The amplified PCR products were electrophoresed in a 1.5% agarose gel, and then stained with etidium bromide (0.05 mg/mL; Sigma Aldrich). The gels were photographed under ultra violet light using Gel Documentation (Bio Rad universal hood II, USA). Molecular size markers (100 bp) were included in each agarose gel. The PCR product was purified (AccuPrep PCR Purification kit Cat No .K-3034-1, Lot No. 10031.) and to determine the sequence, it was sent to the Sequencing Laboratory (Hope Generation Medical Foundation, I.R. Iran).

3.5. PCR Product Cleans up

In order to remove the none-specific fragments of PCR reaction product, electrophoresis on 1.5% Low melting agarose gel have been carried out (80 V for 40 min). The

target band region identified and cut out with scalpel. This piece of gel was transfered into 1.5 μl sterile microtube and the target band was cleaned up according to AccuPrep Gel Purification kit (Bioneer, Korea).

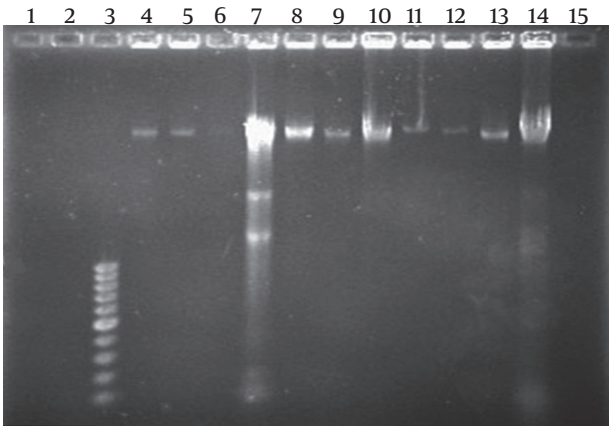
3.6. ELISA Assay

All isolated *S. aureus* strains has been tested by Enzyme immunoassay for the detection of *Staphylococcus* enterotoxins A, B, C, D and E kit (RIDASCREEN SET A, B, C, D, E: Art. No.: R4101 r-Biopharm). All reagents required for the enzyme immunoassay are contained in the test kit and it was carried out based on test direction. In brief, each *S. aureus* strain was inoculated into 5 ml of the BHI-broth (Lot 1.1385000500, Merck Germany) separately and they were incubated at 37°C for 24 hours. Then, they were centrifuged (5000 × g for 5 minutes at 4 × C) and 100 μl of each supernatant was subjected to the ELISA test.

4. Results

From August 2008 to September 2011 a total 350 strains of *S. aureus* were isolated from clinical samples and were identified by bacteriological methods (Gram stain, Coagulase, Manitol fermentation and DNase tests). The frequency of the derived (entP gene containing/total) were from: blood (18/63 strains), CSF (14/51 strains), synovial fluid (3/11 strains), throat swabs (27/97 strains), wounds (25/87 strains), stool (5/24 strains) and genital tract (6/17 strains). The results of DNA extraction by the salting out method represented relative purity and concentration. The sample analysis by Nano-Drap methods revealed the absorption ratio outline of 260/280 nm which was above 1.8, representing the desired purity of the extracted DNA. Agarose gel electrophoresis of DNA extracted by this method showed a favorable and sharp band. As shown in Figure 1.

Figure 1. Agarose Gel Electrophoresis of the Extracted DNA is shown.



Lanes 1, 2 and 15 are negative controls. Lane 3; is molecular size marker 100 bp. Lanes 4-14 shows the DNA extracted from different staphylococcal Strains

The sequences of the primers pair and their analysis are shown in Table 1. As illustrated in Table 1 the F1 R1 and F2 R2 were amplified a 213 bp (Figure 2, A and B) and 700 bp fragments (Figure 3) respectively. In Figure 2, the result of 1.5% agarose gel electrophoresis of PCR products from primer F1 and R1 are shown.

Table 1. The Sequences, Lent, Location and Product Size of Primers are Shown

Primers	Sequences	Length	Location	Product Size
F1	5'-tgt ata tat tgg cgg tgt ctt-3'	21 bp	2011809	213 bp
R1	5'-gct tta agc aat ctt ag-3'	17 bp	2012021	
F2	5'-gtagc GGATCC a gcg aag aaa taa atg gaa t-3'	31 bp	2012115	700 bp
R2	5'-gcgcg AAGCTT aaa aat tgc ata tag ata-3'	29 bp	2011415	

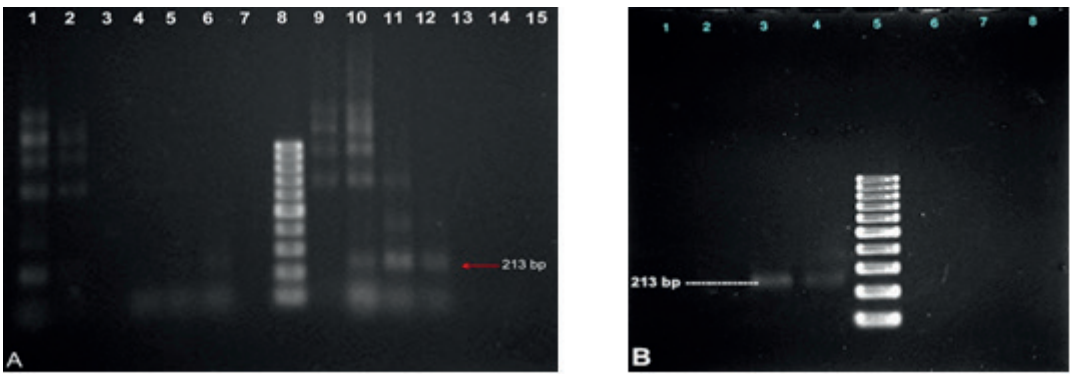


Figure 2. A) Lines 1, 6, 10, 11 and 12 are Shown as a 213bp Fragments (partial entP Gene). Line 8 is a 100bp MW Marker. Lines 3, 7, 13-15 are as Negative Control. B). Lines 3 and 4 Showed the Clean up PCR Product 213bp Fragment and Line 5 is 100bp MW Marker

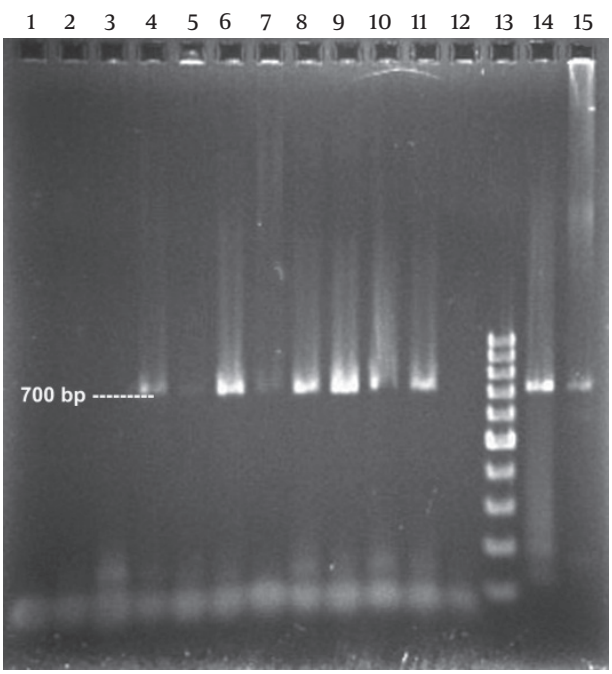


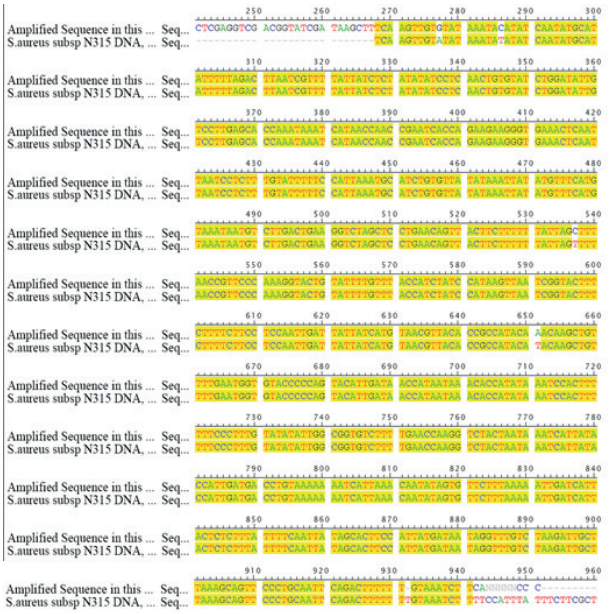
Figure 3. Lines 1, 2, 3 and 12 are as Negative Control. Lines 4-11 and 14-15 Were Shown a 700bp Fragments (Complete entP Gene). Line 13 is a 100bp MW Marker

The results indicate that the PCR method was optimized for entP gene detection. All the 350 isolated strains were studied. The first primer pairs were amplified for 213 bp fragment as well as the second primer pairs which were amplified for 700 bp fragment. The latest fragment were only detected in 98 (28%) out of the 350 strains of *S. aureus* entP gene. The distribution of 98 *S. aureus* strains which carries entP gene were isolated from blood (18 strains), CSF (14 strains), Synovial fluid (3 strains), throat swabs (27 strains), wounds (25 strains) and stool (5 strains) and genital tract (6 strains).

The DNA sequencing of amplicon 700bp fragment of staphylococcal enterotoxin P gene as PCR product was carried out by the Iranian company sequencer (Hope Generation Medical Foundation, I.R. IRAN). The result of multiple alignment of the reference gene (*S. aureus* strain N315 enterotoxin P gene, accession number BA000018.3) with the outcome sequenced PCR product obtained in this study is shown in figure 4. In order to prevent contamination, each of the samples was analyzed separately.

In this study, there was not specific antibody against staphylococcal enterotoxin P. Thus available, the culture supernatant of all strains containing enterotoxin P genes was subjected to ELISA test. The noteworthy finding was that, some strains containing entP gene were also carrying other Enterotoxin genes.

Figure 4. The 700 bp Fragment Amplified (Purified PCR Product) in This Study Was Sequenced and Alignment with Reference Enterotoxin P Gene (accession number BA000018.3) Was Shown.



The First Row Sequence is related to PCR Product Derived From This Research and the Second Row is Reference Gene.

5. Discussion

In this study, we received the enterotoxin P gene sequence from the gene bank and two pair primers were designed and the molecular method PCR were set up. Then, 350 clinical isolate of *S. aureus* strains were assayed. The results indicate that, the total enterotoxin P gene containing strains were 98 (28%) which 79 (22.57%) of them produced other Enterotoxins (A-E). The strains carriers of enterotoxin P gene alone were only 19 strains (5.43%). In addition, ELISA screening tests were conducted with the strains and the results showed that the enterotoxin A was the most prevalent (78% staphylococcal strains were enterotoxin A producer). Sergeev et al. reported that all SEA gene-specific primers can also be used for successful amplification of the SElP gene (20). Our study revealed that the specific primers for SEE gene could be amplified the different clone of SElP gene in clinical isolates. However, SElP was recently described that elicited an emetic response in Suncus murinus (the house musk shrew), but the emetic activity of SElP in human is unknown (21).

The role of staphylococcal enterotoxins (SEs) in different organs of the human body is not exactly clear. However, Staphylococcal enterotoxins (SEs) as superantigens have become a global challenge and receive intensive consideration. There are some reports revealing these

substances have been known contribute to colonization and a broad-spectrum of diseases (22, 23), other than gastrointestinal tract complication. As, literature review revealed that the Staphylococcal enterotoxin B (SEB) is a potent mitogen that elicits life-threatening polyclonal T-cell proliferation and cytokine production at very low level (24). However, the frequencies of infectious disease caused by SEs producer have been reported differently. In addition, a group of staphylococcal virulence factors called the superantigen-like proteins (SSL) has been reported (25).

Some investigators showed that the level of antibody specific to the *S. aureus* enterotoxins in carriage in later stages of pregnancy has been raised. They concluded that over 89% of women and infants have some protective antibody to the toxins, and antitoxin IgG levels are higher in cord blood samples compared to antenatal samples and some infants lack protection and could be at risk of toxin-induced disease (26). Normanno et al. demonstrated that Enterotoxins of *S. aureus* are able of inducing a cytopathic effect in cell lines (27). Najera-Sanchez et al. showed that the most frequently produced types being SEA and SED. They found that the Levels of correlation between the presence of genes that encode for the production of SEs (as determined by polymerase chain reaction) and the expression of these genes (as determined by the indirect ELISA) were 100% for SEA and SEE, 86% for SEC, 89% for SED, and 47% for SEB (27).

These facts have allowed us force to extensive analysis of the distribution of *S. aureus* genetics elements encoding enterotoxin-like superantigens (SEls) to prevention of future complication. NCBI-Blast results from this study suggest that, the specific primers amplification ent E gene were able to amplify the fragment which shows 100 % homology with different clone of staphylococcal Enterotoxin-Like Toxin Type P and only 81% similarity with reference gene. While, the specific primers for entP gene was amplified a fragment gene (700 bp) showed 100% homology with reference gene (enterotoxin P gene, accession number BA000018.3).

In conclusion, the genetic similarity among the Enterotoxin A, E and P was found to be very high. Only 5.43% of *S. aureus* strains were carriers of Enterotoxin P gene alone. In addition to Enterotoxin P gene, 22.57% of strains were carrying other Enterotoxin gene. Therefore, this may suggest that the same mechanisms of pathogenic action in body site. However, Staphylococcal enterotoxin-like proteins as superantigens has necessitated intensive further investigation to determine frequencies of distribution.

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Authors' Contribution:

Ramezan Ali Ataee, Rahim mansuor Khanshan and Mohammad Hosein Ataee developed the original idea and the protocol, abstracted and analyzed data, wrote the manuscript. Ramezan Ali Ataee, Mojtaba Hedaiati ch and Mohammad Hosein Ataee contributed to the abstracted data, and prepared the manuscript.

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