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Heterologous Expression of Staphylococcal Enterotoxin E Gene

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One of the main virulence factors of *S.aureus* is a superantigene, named enterotoxin type E. The entE gene (693 bp) was isolated from the native strains of *S. aureus*. It was amplified with PCR and sequenced. The entE gene was cloned into a pET-28a expression vector. Then, the recombinant plasmids were electroporatively transformed into *E. coli* Rosetta BL-21(DE3) strain and was expressed. The recombinant entE was purified and confirmatory tests were performed. The results showed the specific pattern and it¢s similarity with the reference entE gene. The recombinant protein (~30Kda) was confirmed by western blotting. RFLP pattern, sequencing and western blotting of the gene product confirmed the entE gene. Therefore, the method of cloning and expression of entE gene was set up and paving the way for the production of specific antibody and achievement of rapid diagnostic method for SEE.

Key words: *Staphylococcus aureus*, Recombinant protein. Enterotoxin E. Cloning. Expression.

Staphylococcus aureus is a ubiquitous bacterium, an important pathogen associated with numerous human and animal diseases[1]. *S. aureus* produces single-chain Staphylococcal enterotoxins (SEs), which decrease the immune response of a colonized host by strike specific subsets of T cell[2, 3]. *S. aureus* infections evince health and economic problems throughout the world[4].

SEs are associated with food poisoning[5] and consumption of small amounts of each toxin elicits emesis and diarrhea within ~4h [6]. The Staphylococcal enterotoxin A (SEA) is most commonly associated with food poisoning[7]. However, various populations are naturally exposed to Staphylococcal enterotoxins throughout the world[8]. The SEs draw out enteric effects and inflammatory responses such as leukotrienes and prostaglandins[9].In addition, SEs have synergistic effects with some viral infections[10]. According to serological criteria, SEs are classified into 21 major groups[11,12]. The investigators compared the amino acid sequences of SEA, SEB and SEC1 and demonstrated that these proteins are not only related to each other but also to streptococcal pyrogeneic exotoxins[13,14,15]. The entA and entE gene *S. aureus* strains have high homology with each other. Some investigators reported that the entE gene has %84 nucleotide sequence homology with the entA gene and %80 with the entP gene[16]. Comparison of amino acid sequence data obtained from nucleotide and peptide analyses suggests that SEE is a precursor contained of 257 amino acid residues (molecular weight; 29.358 KDa). This peptide is synthesizes and processed to produce a mature form of 230 amino acid residues with the molecular weight of ~26.425 KDa [17]. However, SEE is more closely related to SEA and SEP than to SEB and SEC1[18-19].

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Moreover, in recent years, it has been shown that some strains of coagulase-negative *Staphylococcus aureus* are able to produce considerable amounts of enterotoxins. These enterotoxins appear to play an important role in causing various diseases[20]. Hence, to achieve Staphylococcal enterotoxins detection methods can help to the improvement of diagnostic tools and health promotion. Therefore, production of antibody for detection and treatment of food poisoning by SEE is highly essential. The purpose of this study was screening and isolation of the entE gene and then cloning and expression of recombinant enterotoxin E and using confirmatory tests such as sequencing, RFLP (Restriction Fragment Length Polymorphism) pattern and western blotting.

MATERIALS AND METHODS

Bacterial strains, vectors and media

Staphylococcus aureus native strains that produce enterotoxin E were isolated from the clinical samples. Two *E. coli* strains; DH5α and BL-21(DE3) pLysS and Rosetta BL-21(DE3) pLsS, were obtained from Invitrogen and Novagen (USA). Plasmid pET-28a as expression vector was purchased from Novagen (USA). The bacteria were cultured in LB broth or on the IPTG-XGal-LB agar (fermentas, USA) with or without 40µg/ml Kanamycin (Sigma, USA) and 100µg/ml Ampicilin (Roch, Germany).

The 35 strain of *S. aureus* as producer enterotoxin E were early screened by beneficial molecular method PCR and ELISA Kit in the Therapeutic Microbial Toxin Research Center of Baqiyatallah Medical Sciences University (Tehran. I.R Iran). *S. aureus* morphology was determined by light microscopy after Gram staining and the cultures were approved with catalase, oxidase and coagulase tests. Then they were preserved and stored at -20°C.

Extraction of genomic DNA

The 24hour culture of *S. aureus* bacteria were added into 1.5 ml micro centrifuge tubes, washed once with phosphate-buffered saline (pH 7.2) and sedimented by centrifugation at 9000×g for 3min. Genomic DNA was extracted by AccuPrep ® Geneomic DNA Extraction Kit (Bioneer, Korea). Then the DNA pellet was suspended in buffer TE (10 mmol/L Tris-HCL, 1 mmol/L EDTA pH: 8.0) and stored at -20°C.

Synthetic primers

Based on the Gene Bank of strain M21319.1, a single primer pair was designed and applied to amplify the S. aureus entE gene target fragment. The primers had a BamHI site incorporated into the 5' end and a HindIII (both from Fermentas, USA) site at the 3' end. The primers sequences were as follows: Forward: 5'- gtagc GGATCC agc gaa gaa ata aat gaa a -3' and Reverce: 5'- gcgcg AAGCTT tca agt tgt gta taa ata c -3'. These primers were able to amplify a 693bp fragment which was without signal peptide. PCR was performed in a standard enzyme expand high fidelity by using C1000 Bio-Rad Thermo cycler system (USA). The PCR was performed in a 25µl reaction mixture by using 1.3 unite/µl Taq DNA polymerase, 1.5mM 0.8mM $MgCl_2$, 100mM/µl dNTPs, 10uM/µl from each primer and 5ng/µl DNA template. The amplification conditions were 4 min at 94°C for the primary denaturation, 40 sec at 94°C for the secondary denaturation of the target DNA, then annealing at 55°C for 55 sec, extension at 72°C for 1 min and final extension at 72°C for 7min that 35 cycles. The PCR products were analyzed by electrophoresis on 1.5% agarose gel (Sigma LTD) and staining solution containing 0.1 g of ethidium bromide per ml in TBE Buffer. The gel electrophoresis was visualized under UV light and photographed.

Cloning and construction of recombinant plasmids

The PCR products were purified by AccuPrep PCR Purification Kit (Bioneer, Korea). The purified products were ligated into the compatible sites of the pBluescript II (Fermentas, USA) by using T4 DNA ligase (Fermentas, USA) at a molar ratio of 3:1 at 22°C for 1hours and then at 16°C for 12hours. After that, these were transformed into *E. coli* DH5α (Novagene, USA), and cultured them onto the LB agar–Amp (100µg/ml)-XGal-IPTG (Fermentas, USA) medium. The white clones of the pBluscript/entE were selected and identified by PCR and restriction enzyme digestion was performed. Finally, they were sequenced by a commercial facility using universal forward and reverse T7-promoter and T3-terminator primers (GenFanAvaran Co. Branch TAG Copenhage, Denmark).

Extraction of recombinant plasmid

The single bacterial colony (*E. coli* DH5α/

pBluscript/entE) was picked up and inoculated in 5 ml LB broth containing 100µg/ml of Ampicillin. Then it incubated at 37°C overnight. The recombinant plasmids were extracted according to the manufacturer's instructions of AccuPrep® Nano plus plasmid Mini Extraction Kit ((Bioneer, Korea), and identified by the PCR. Then restriction endonuclease digestion was carried out. The entE gene was amplified from the cloning vector (pBluscript/entE) and sub-cloning of the entE gene fragment was performed in pET-28a. In fact, the plasmids containing the entE gene fragment were purified according to manufacturer's instructions DNA Purification Kit (Bioneer, Korea). Then restriction enzyme digestion was performed and the entE gene was ligated with pET-28a at a molar ratio of 3:1 at 22°C for 1hours and then at 16 °C for 12hours. The recombinant plasmids were transformed into *E. coli* DH5α hosts. The recombinant hosts were inoculated in 5 ml LB agar containing 100µg/ml of Ampicillin, then incubated at 37°C overnight. The *E. coli* DH5α hosts containing pET-28a/entE clone were selected and identified by the PCR and restriction enzyme digestion. Finally, they were sequenced by a commercial facility using universal forward and reverse T7-promoter and T7-terminator primers (GenFanAvaran Co. Branch TAG Copenhage, Denmark).

Cloning and construction of expression plasmid

The plasmids containing entE gene fragments were purified according to the manufacturer's instructions of DNA Purification Kit (Bioneer, Korea). After enzyme digestion, they were transformed into competence cells of *E. coli* DH5α and *E. coli* Rosetta BL21 DE3 by electroporation. Then the transformants of pET-28a/entE were selected on LB agar plates containing 40ug/ml Kanamycin[21] and identified by the PCR and restriction enzyme double digestion. The *E. coli* cells harboring expression vector pET-28a/entE were grown in LB medium supplemented with Kanamycin (40 µg/ml) and Chloramphenicol (34 µg/ml) at 37°C until obtaining an OD 600 = 0.6 - 0.8 for induction. Then IPTG (Sigma, USA) was added into the above culture at a final concentration of 1 *m*M and the culture was grown at 37°C for 5hours. then, the recombinant bacterial cells were harvested and suspended in lyses buffer (NaCl 0.5 M, PMSF 1 mM, EDTA 10 mM, Triton X-1001% (v/v), 20 mM Tris-HCl, pH: 7.5). Then it was frozen and thawed three times or ultrasonicated on ice in the presence of 1mM PMSF (Sigma). The recombinant entE proteins were centrifuged in 14000×g for 20min at 4°C and were collected for SDS-PAGE which was done by Bio-Rad protein cell vertical slab gel apparatus[22].

Endonucleas restriction digest

After purification of the target gene in the recombinant plasmids, they were digested with DraI and RsaI restriction enzyme (Fermentas, UAS); each enzyme in volume 2µl (10unit/µl) was fast digest. The reaction was performed in 40µl of solution, containing recombinant target gene (100ng/10µl), 10X Green buffer fast 4µl and 24µl of D.D.W. The mixtures were incubated for 30min at 37°C and then for 10min at 80 °C for enzymes inactivated. The outcome product was electrophoresed and visualized by 1.5% agarose gel.

Antibody

In this study, we used the Rb Ab *Staphylococcus* Enterotoxin E 500µg (1mg/ml), which was purchased from Abcam USA (Ab 15922 500 lot 713307).

Purification of recombinant enterotoxin E

The purification of recombinant enterotoxin E was carried out according to the manufacturer's instructions of V8550 MagneHis™ Protein Purification System (Promega, USA). The purified protein was subjected to SDS-PAGE[23].

Western-blot analysis

Western-blotting was performed according to the Bummatte's method[24].

RESULTS

PCR amplification

As shown in Fig. 1, the entE gene of *S. aureus* was amplified by the PCR method. The PCR products were electrophoresed and visualized by 1.5% agarose gel. The results revealed that the size of entE gene was ~700bp DNA fragment.

Identification of recombinant vector by PCR and restriction digestion

The results confirmed that the recombinant plasmids were extracted from recombinant *E.coli* DH5α bacteria. These plasmids were used as template to amplify by PCR under the conditions of mentioned before (Fig. 2a). Double

digestion of the recombinant plasmids with BamHI and HindIII restriction enzymes showed that the ~700bp fragments were released (Fig. 2b). The PCR and digestion products were visualized by 1.5% agarose gel electrophoresis on acceptable manner.

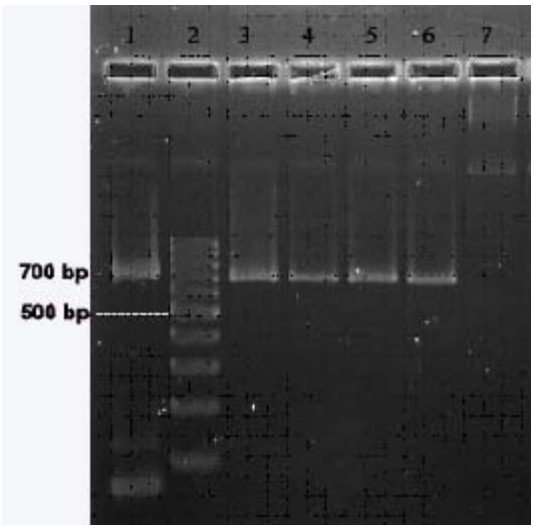

Fig. 1. 1.5% Agarose gel electrophoresis patterns showing the PCR amplification. Lane 1 positive control for SEE (native strain), Lane 2: standard molecular size marker 100 bp (Fermentas, USA), Lanes 3-6: SEE (~700bp), and Lane 7: negative control for SEE

RFLP with restriction enzyme

The results of RFLP pattern with RsaI and DraI restriction endonucleases showed that the entE gene is particular and differs from the entA and entP genes, which have high homology with each other. DraI digested the recombinant ent E gene at 234 bp location and generated two fragments; 153 bp and 540 bp, respectively, with the same restriction pattern of the entA gene. While, the RsaI digested entE gene at 394, 446 and 485 locations that produced 4 fragments of 313 bp, 52bp, 40 bp and 210 bp, which had different restriction pattern from that of the entA gene.

Western-blot analysis

The results of Western-blotting showed that the method of the Burmnette[24,23]could confirm the recombinant proteins following the protein separation by SDS-PAGE. The proteins bond was transferred into nitrocellulose paper by Trans Blot SD cell system (Bio-Rad, semi dried). The electro blotting was performed within 30 min at 15 mV at room temperature. Then, the non-specific binding was blocked with 3% BSA and the nitrocellulose papers were incubated for 2 hours with 1:10000 dilution of the primary antibody in 0.5% BSA. After being washed and incubated with goat anti-IgG rabbit (secondary ab) horseradish peroxidase (HRP) conjugate antibody (Bio-Rad) at 1:8000 dilution, the nitrocellulose papers were stained with POD (o-phenylenediamine, Roch), (Fig. 4).

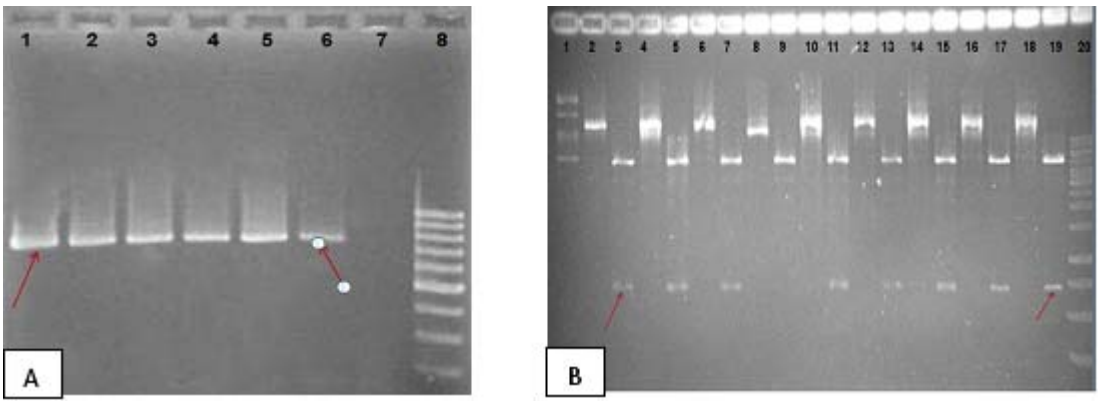

Fig. 2. PCR Recombinant plasmid vector (A), and double-digestion recombinant plasmid (B). part A: Lanes 1-5; shows the entE recombinant from pET-28a plasmid, Lane 6; positive control for entE gene (Native strain), Lane 7; negative control for entE gene and Lane 8; standard size marker 100 bp (Fermentas, USA). **Part B**: Restriction double-digestion of positive recombinant plasmid: Lane 1; pET-28a, Pair lanes; extracted plasmid, Singular lanes; digested same plasmid and Lane 20; standard size marker 1kb (fermentas, USA). The results indicated that recombinant plasmid contained the objective gene (red array). At the same time, it was successful in transforming the recombinant plasmid into *E. coli* DH5α

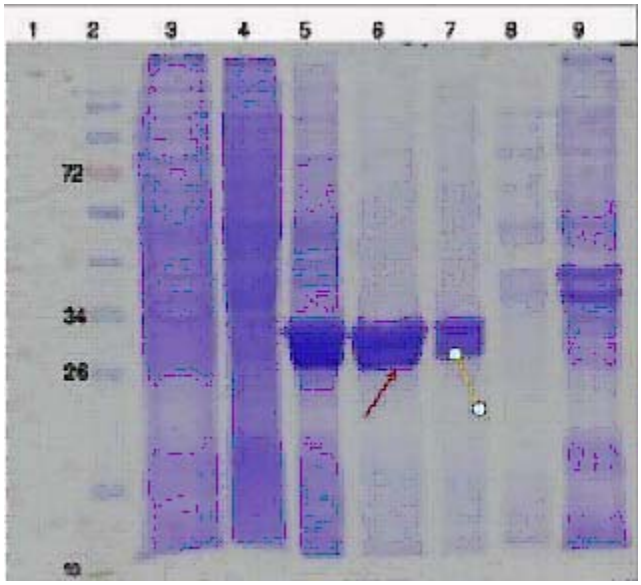

Fig. 3. SDS-PAGE of recombinant and purified protein SEE. The gel was stained with Coomassie blue G-250 (Sigma). The samples were resuspended directly in PBS 1%, added into SDS loading buffer and boiled for 10 min. Lane 1; pET-28a vector, Lane 2; protein molecular weight standard (fermentas USA), Lane 3; supernatant after sonication (Crude), Lane 4; supernatant after sonication and centrifugation, Lane 5; recombinant protein after 4h since induction by IPTG 1mM, Lane 6; recombinant protein purified by Ni2- Sepharose resin red arrow, Lane 7; recombinant protein purified by promega His-Tag system yellow arrow, Lane 8; recombinant *E.coli* cell before induced by IPTG 1mM, and Lane 9; *E.coli* Rosetta BL21-DE3 non induce

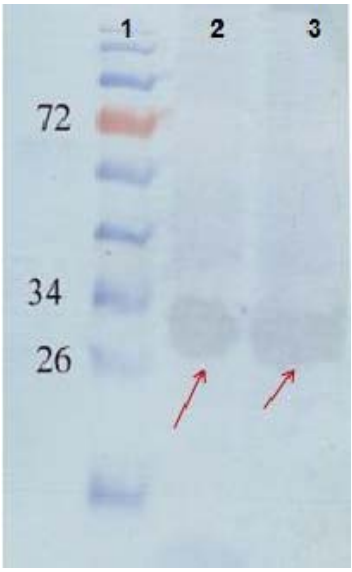

Fig. 4. Western bloting recombinant pureed SEE protein with anti SEE polyclonal mono specific antibodies. Lane 1, protein molecular weight standard (Fermentas USA). Lane 2 and 3 shown the western blot rSEE which induced with IPTG. HRP-conjugated anti-rabbit IgG (1:8000) and OPD were used.

DISCUSSION

There is evidence that high genetic similarity is among the Staphylococcal enterotoxins genes. It is noticeable that the highest similarity 84% is among between the entE and entA genes[16]. Thus in this case, sequencing result may not be convincing alone. Hence, as the results of our study revealed, in addition to sequencing, restriction enzyme digestion (RFLP), ELISA test and western blotting could verify confidential differentiation among the above genes. Our results were similar to those of the previous research[25]. Some investigators showed differences between the entE, entA and entP sequences using the RFLP and western blot or gel double-diffusion procedures[26]. In this research, the RFLP pattern and electrophoresis showed that the sites of restriction enzyme inside the gene are completely different from those of the entA gene. In addition, the western blotting confirmed the entE gene product. This is the second research that has attempted to conduct the cloning and expression of the entE gene. The first study was done by Couch *et al.*,[16]. The

similarity of the two studie¢s results was toward sequencing and cloning of the entE gene and also the similarity of the molecular weight of the expressed protein. The discrepant of these studies was on the use of plasmids vectors and analyses procedure. In our study, two types of plasmid vectors (pBluscript and pET-28a) were used for cloning and expression separately. However, Couch et al used phages for non-productive strains and produced enterotoxin E. In addition, there is a report that verified the production of toxin by using DNA blot hybridization. In this investigation, we produced high quality and purity recombinant Staphylococcal enterotoxin E for immunization of animal models and for specific antibody production in future research.

Our finding showed that the extracted entE gene is distinct from entA and entP. In addition, the entE DNA was digested with BamHI and HindIII. The fragments were separated by electrophoresis through a 1.5% agarose gel. Then they were ligated to double digest pET-28a DNA. The recombinant DNA was transferred into *E. coli* Rosetta BL21-DE3 by electroporation method. Kanamycin (40µg/ml) resistant transformants were selected and purified. In nature, the Staphylococcal enterotoxin E (SEE) is an extracellular protein, which is encoded by entE gene. This protein consists of 257 amino acid residues with the molecular weight of 29.358 KDa, which is apparently processed by proteolysis to a mature form with a molecular weight of approximately 26.425 KDa[16,17]. In this research by adding 6 His-Tags to the end of the protein by pET28a, the recombinant molecular weight of SEE was obtained as ~ 30KDa (Fig. 3).

The nucleotide sequence, RFLP and western blot of the product entE gene (Fig.4) were compared with those of the entA, entB, entC, and entP genes (data not shown). Variation between the obtained sequence and standard sequence may be pertaining to the used strain, which was a native strain that was collected from the clinical samples. However, given the need to achieve rapid diagnostic method for Staphylococcal enterotoxins and producing the specific anti- serum for food poisoning case treatment, setting up the method to clone and express the recombinant production of small amounts of enterotoxin E is very important, because of the *staphylococcus aureus* naturally produces small amount of SEE in its native form. A recent study has shown that food poisoning caused by SEE is epidemic in some countries and also superantigeneic properties demonstrated its importance[27]. However, the recombinant protein SEE in this study was estimated by SDS- PAGE, and the results indicate that, it was a ~30 kDa protein and reacted with specific antibody in the Western blot, confirming it as Enterotoxin E. Antibodies are usually sensitive indicators for diagnostic conformational change in the enterotoxins. However, conformational change in the structure of enterotoxins perhaps causes the loss of emetic and serological activities[17]. In summary, restriction enzyme pattern and western blotting analysis of the recombinant SEE showed it's accuracy and high quality. Accordingly, we suggest that the recombinant SEE can be used for immunization of animal models for produced specific antibodies.

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