Standardizing Molecular Detection of the entC Gene of Staphylococcus Aureus Isolated from Human Infections and Sequencing it

Ataee RA.*,1 PhD, Kamali M.3 PhD, Ranjbar R.4 PhD, Karami A.4 PhD, Ghorbani M.2 MSc

1Department of Microbiology and Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
2Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
3Nanothechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
4Molecular Biology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

Abstract
Aims: The goal of this research is standardizing molecular detection of the entC gene of Staphylococcus aureus isolated from human infections and determining its sequence.
Methods: At first, the primer was prepared using the standard gene in the gene bank. Then, the molecular PCR method was set up to identify the entC gene in the Staphylococcus aureus bacteria extracted from human specimens (300 strains). The PCR product was sequenced and compared with the standard gene. Also, the ability of all entC gene carrier strains to produce enterotoxin C was tested using an ELISA kit.
Results: The results showed that the molecular technique of PCR had been well set up with various primers, because, the first and second primer pairs easily amplified a 102bp and a 1223bp fragment, respectively. A comparison of the sequence of the 1223bp fragment with the reference gene showed 99% compliance. The translated form shows a difference in position 218 only where alanine had replaced valine. The results of the ELISA test for assessing the toxigenicity of all the strains carrying the entC gene showed that 37% of the strains contained the gene.
Conclusion: Not only coagulase positive Staphylococcus aureus strains but also coagulase negative strains produce superantigenic enterotoxins. This research provides a simple and fast method of detecting toxigenicity of the Staphylococcus aureus bacteria and a method of standardizing detection of enterotoxin C-producing strains, because the detection of superantigenic enterotoxins can be a very valuable help intreating patients infected with them and preventing further complications.

Keywords: Standardization, Staphylococcus Aureus, entC, PCR

Introduction
Staphylococcus aureus is the most common opportunistic pathogenic organism which is reportedly found in 30% of healthy carriers and which produces toxin in 50% of the isolated human strains [1, 2]. In recent years, the toxigenicity of Staphylococcus aureus has attracted the attention of scientists for different reasons. A) some coagulase negative strains, too, have been able to generate enterotoxins causing food poisoning [3]. This has reduced the validity of the coagulase test in determining the toxigenicity of Staphylococcus aureus. B) all toxins generated by these bacteria show a superantigenic activity which can make for various effects on the host [4]. C) this type of bacteria is reported to have connections to non-digestive diseases [5, 6]. D) researches have shown that the Staphylococcus bacteria which produce toxic shock syndrome toxins always creates enterotoxin C [7, 8].E), According to some researches,
Staphylococcus enterotoxins can be used as an antineoplastic drug [8-10] and as an accelerating agent in treating broken bones of animals [11]. Although the prevalence of the toxigenic strains of this type of bacteria has not been precisely specified, according to many reports, these bacteria have been isolated from dairy products [12] and different infections [13]. So far, 20 types of Staphylococcus aureus antigenic enterotoxins have been introduced and named [14]. Enterotoxin C is one of the five most widespread enterotoxins involved in food poisoning and the toxic shock syndrome. It has also been suggested that Staphylococcus enterotoxin C (SEC) has a role in deteriorating infection in experimental animal models. This toxin has three biotypes: SEC1, SEC2 and SEC3. Enterotoxins are notorious for causing food poisoning in vitro, which means eating contaminated food can result into food poisoning. This fact reveals that these Enterotoxins are resistant to digestive enzymes. Although the superantigenic mechanism of these enterotoxins and their function in the digestive system are known, exactly how they affect the other parts of the body is not yet clear. It is believed that the SEC-coding gene is located on the plasmid carrying the antibiotic-resistant gene [15], which accounts for its possible prevalence and dissemination. This is why enterotoxin C is the most widespread food-poisoning agent in dairy products after SEC. Although the relationship between entC and entA is not yet clear, some of the strains producing entC generate other enterotoxins as well as the toxin responsible for the toxic shock syndrome at the same time. This is why food poisoning epidemics are common [16].

Because of the widespread distribution of Staphylococcus bacteria in nature and their ability to produce different types of enterotoxins, all sorts of food are exposed to contamination with enterotoxins. Even aquatics such as shrimps have been reported to be contaminated with Staphylococcus enterotoxins [17]. It should be noted that enterotoxins are not only created by coagulase-positive Staphylococcus bacteria but, as recent studies have shown, also by coagulase-negative ones [18, 19]. Although the role of enterotoxins in causing non-digestive diseases is not clear, the presence of Staphylococcus enterotoxins genes has been reported in patients suffering from atopic dermatitis, psoriasis and erythema [20], which reveals the possible role of enterotoxins in some diseases. Malam et al (1992) have referred to the sudden death of babies as a result of accumulation of Staphylococcus enterotoxins in kidneys [21]. It is worth noting that the sequence of the Enterotoxins- coding genes vary in different geographical areas [22]. Yet, it is not clear whether or not this variation in the gene sequence causes changes in the sequence of amino acids and specific antibodies. In any case, since identifying pathogenic Staphylococcus aureus depends on coagulase tests, and because coagulase-negative Staphylococci can produce superantigenic enterotoxins, the detection of the toxigenicity of these bacteria while carrying out coagulase tests can be of great help in providing the accurate diagnosis and preventing further complications. Accordingly, it is unavoidably necessary to standardize the detection of enterotoxin-producing strains, especially for epidemiological studies. In addition, it has been reported that there is a relationship between the ability to produce enterotoxins and resistance to methicillin [23, 24], but it is not yet clear whether resistance to methicillin makes for the induction of enterotoxins or vice versa. In view of the above explanations, the aim of this study is to standardize the method for detecting the SEC gene isolated from clinical specimens.
Methods

Materials: The molecular material needed for this research was purchased from an Iranian company called Sinagene, which is a provider of Fermentase products in Iran. The culture media were bought from Arya Vajd Company, which sells German Merck in Iran. The PCR product extraction kit (DNA Extraction; Cat. No. K-3032, Lot No. 10032) was bought from Takapoozist, which sells Bioneer products in Iran. The kit for detecting enterotoxins was purchased from Rocket International, providing the r-Biofarm products.

Methods: In this experimental research, a method was set up for detecting enterotoxin C (entC) genes in Staphylococcus aureus strains isolated from patients. Three hundred strains of Staphylococcus aureus strains isolated from clinical specimens were studied and the strains bearing entC genes were examined with RIDASCREEN SET A,B,C,D,E: Art. No.: R4101 in terms of producing enterotoxin C. Also, using immunoblot analysis and monospecific polyclonal antibodies (Rb PAb Staphylococcus enterotoxin C 500 Ug (1mg/ml), Ab 15897, Abcam) the existence of enterotoxin C was confirmed.

Criteria for choosing bacterial strains were as follows. The bacteria isolated from the culture of infected specimens with gram-positive cocci in grape-like clusters, positive catalase, positive coagulase, positive DNase and ability to grow in mannitol salt agar media were included in this research. Thus, 300 strains of Staphylococcus aureus studied were isolated from superficial infections, blood, stool, throat culture, urine and spinal fluid. In order to preserve the strains, 20% glycerol was added to their 18-hour culture and they were kept in a freezer at a temperature of -20 degree Celsius.

Bacteria culture and genome extraction: The genomes were extracted using a slightly-changed slating-out method [25]. The colonies of each bacterium were separately inoculated into LB culture media and after 24 hours of incubation, 1ml of it was put in 2ml sterile tubes and centrifuged for 10 minutes at g x 5000. The supernatant fluid was discarded and the remaining part was added to and completely mixed with the cellular sediment of a 400μl STE buffer solution. 125μl of a 2% SDS solution and 250μl of a 3-molar sodium stat solution were added to it and inverted for 10 times to complete mixing. The material was then centrifuged for 5 minutes at g x 5000. At this point, the genome entered the supernatant fluid. This fluid was then transferred into a sterile tube where, after adding 750μl of cold isopropanol (or absolute ethanol) to it, the materials were slowly mixed and the mixture was kept in the freezer at -20 degrees for one hour and sometimes overnight. Then, the solution was centrifuged for 5 minutes at g x 12000 and 4 degrees Celsius, and then the supernatant fluid was discarded. The sediments were completely dried and 750μl of cold 70% ethanol was added to it and after being frozen for 1 hour at -20 degrees Celsius was centrifuged for 5 minutes at g x 12000 and 4 degrees Celsius. Again, the supernatant fluid was discarded and the sediment was thoroughly dried. Fifty μl of a TE buffer solution was added to the sediment and the product was kept at 35 degrees Celsius for 45 minutes. Then, the DNA concentration was measured using a NanoDrop unit. One μl of the resulted genome solution was put as a template in special PCR tubes and frozen at -20 degrees Celsius. Whenever necessary, each of the tubes was taken out of the freezer and after preparing gradients, were used for PCR purposes.

Primers: Two pairs of primers were utilized in this research. After studying many articles, one pair of primers, which had been frequently used by researchers and had the sequence of...
F1: 5’TGTATGTATGGAGGTGTAAC-3’ and R1: 5’-AATTGTGETTCTTTATTTTCATAA, was chosen [17]. Using the sequence of the reference entC gene, the other pair was taken from the Gene Bank: AB084256.1 and designed as the specific pair using the Alel ID software and with the sequence F2: 5’-GGAATGTGGATGAAGGAG-3’ and R2: 5’-TATGGACACAATGATACTGG-3’. These pairs were then synthesized by Sina-Gene Company. According to bioinformatics analyses, the primer pair of F1 and R1 multiplied a 102bp fragment and the primer pair of F2 and R2 multiplied a bp 1223fragment.

**Polymerase chain reaction:** A polymerase chain reaction was done to multiply the bp102 fragment based on the protocol given in Table 1.

| Table 1) Conditions of Carrying of the PCR for Detecting the Complete entC Gene with a 102bp in Staphylococcus Strains Isolated from Clinical Specimens |
|---|---|---|---|---|
| **Master Mix** | **PCR Condition reaction** | **Reagents** | **Step** | **Condition reaction** | **Time** |
| D.D. Water | 18.6μl | Denaturation primal | 95 C | 3 min |
| Template DNA | 15 ng/0.5μl | Denaturation | 94 C | 30 sec |
| Buffer 1X | 15 ng/0.5μl | Annealing | 53 C | 30 sec / 35 cycles |
| MgCl2 | 1 mM/1μl | Elongation | 72 C | 5 min |
| dNTP | 0.2 mM each/0.7 μl | Final Extension |  |  |
| F1- Primer | 0.3 mM/1 μl | Incubation | 4 | 5 min |
| R1- Primer | 0.3 mM/1μl | Taq enzyme | 2 unit/0.3μl |  |
| Total | 25 | | | |

| Table 2) Conditions of Carrying of the PCR for Identifying the Complete entC Gene with a 1223bp in Staphylococcus Strains Isolated from Clinical Specimens |
|---|---|---|---|---|
| **Master Mix** | **PCR Condition reaction** | **Reagents** | **Step** | **Condition reaction** | **Time** |
| D.D. Water | 16.9μl | Denaturation primal | 95 C | 4 min |
| Template DNA | 15 ng/1μl | Denaturation | 94 C | 40 sec / 35 cycles |
| Buffer 1X | 2.5μl | Annealing | 60 C | 40 sec / 35 cycles |
| MgCl2 | 1 mM/1.5μl | Elongation | 72 C | 6 sec / 35cycles |
| dNTP | 0.5 mM, each 0.7μl | Final Extension |  | 10min / 1 cycle |
| F1- Primer | 0.3 mM/1 μl |  |  |  |
| R1- Primer | 0.3 mM/1μl | Taq enzyme | 2 unit/0.4μl |  |
| Total | 25 | Incubation | 4 | 5 min |
In order to optimize the PCR conditions, a temperature gradient (of 46–56 °C), magnesium chloride concentration gradient (of 5.1 – 8.0 mM) and a template concentration gradient (of 10 -100 ng/ml of the extracted genome) were utilized. This reaction was carried out using the Thermal Cycler, C1000 manufactured by Bio-RAD. The PCR product was electrophoresed with agarose gel of 1%-1.5%, stained with ethidium
bromide and examined under ultraviolet light (of a Gel Doc). In order to determine the sequence, the certain band was purified using a PCR product extraction kit and was sent to an Iranian company called Nasl-e Omid. Since it is possible for the part making up the bp102 fragment to be shared by other enterotoxins, primers F2 and R2 were designed and used which multiplied complete entC genes and the bp1223 fragment. In fact, the PCR was run to multiply the bp1223 fragment based on the protocol given in Table 2. A temperature gradient (of 53 – 62.9 C), a magnesium chloride concentration gradient (of 1 -2 mM) and a template concentration (of 10 – 100 ng/l of the extracted genome) were used.

**Detection of toxigenecity:** All the strains which were confirmed as carrying the entC gene using the molecular PCR method were inducted into the BHI-broth culture medium and centrifuged after 24 hours of incubation. The supernatant fluid was then checked for the presence of entC using an ELISA kit. Also, as a quality control process, the toxigenicity of the non-entC-carrying strains were tested using ELISA. In order to carry out the ELISA test, a specific kit of detecting 5 most widespread enterotoxins of Staphylococcus aureus (RIDASCREEN SET A,B,C,D,E: Art. No.: R41011), manufactured by the German company of r-Biofarm was used and the company instructions were followed.

**Results**

The results of phenotype testing on all strains confirmed that they were all Staphylococcus aureus bacteria, and thus 300 strains were included in the study. The results of extracting the genomes of all the strains using a slightly-changed salting out method were comparable to those of commercial kits. Figure 1 shows the concentration measurement and electrophoresis of the genome extracted from the Staphylococcus aureus bacteria.

The results of optimizing the multiplication of a portion of an entC gene are shown in Figure 2. Picture A depicts the PCR with a temperature gradient (of 46-56 C) and an MgCl2 gradient (of 5.1-8.0 mM). The multiplication of the entC in non-optimum conditions generated a band in the 102bp area. In picture B, by producing optimum temperature conditions (a gradient of 47-57 C), the PCR product made a strong band in the 102bp area in wells 5 and 6. In picture C, using the optimum temperature conditions (51.9 C) an optimum (2 millimolar) concentration of MgCl2 and the primers, the chosen bands were amplified and the other bands were eliminated. Picture D depicts the purification of the PCR product and its preparation for sending to be sequenced. Since previous researches have reported the entC gene to be 613-1095bp long, tracing the 102bp fragment in various strains of Staphylococcus might not properly represent entC. Accordingly, and based on the existing sequence in the gene bank, primers were designed which could multiply a 1223bp fragment with 128 bases more than the given sequence (Figure A3). By providing the optimum conditions, as Table 2 reveals, the PCR came to be the 1223bp fragment. Picture B in Figure 3 shows the purified PCR product which was prepared to be sent for sequencing.

**The entC sequencing results:** A comparison of the gene sequencing of this research with the entC gene of the gene bank (GeneBank: X05815.1) is given in Figure 3. As shown in the Figure, in base 104, amino acid A has replaced T; in base 636, amino acid A has replaced A; in base 659, amino acid C has replaced T; in base 980, amino acid T and in base 1049 amino acid G have been replaced. In this research, primers were used which had multiplied a fragment of about 1223bp. A comparison of the PCR product sequencing
reveals that bases 91-1056 match and there is no match in 6 sequences only, while the translated sequences showed a mismatch in one amino acid only (Figure 4).

**The results of ELISA test for confirming the production of enterotoxin C:** The results of ELISA testing on the strains with and without entC are shown in Figure 6. As shown, columns 9 and 10 include the strains that produced enterotoxin C only. Columns 1, 2, 4 and 6 show the strains which produced enterotoxin A only. Columns 3 and 7 are populated by the strains which produced no enterotoxins at all. Column 8 concerns the strains that produced a little of enterotoxins B and C and much enterotoxin E. Columns 11 gives the strains that produced much enterotoxin A but little of enterotoxins D and E. Column 12 contains the strains that produced enterotoxins C and E. The PCR with the strains studied in columns 8, 9, 10 and 12 confirms the existence of entC in them. In this picture, rows F and G are negative controls and row H is a positive control related to the standard enterotoxin (2-10 ng/ml) in the ELISA kit.

The results of studying 300 strains of Staphylococcus Aureus using the molecular PCR showed that 37% of the strains contained the entC gene. But ELISA revealed that only 11% of the strains produced the entC gene exclusively while the remaining 26% of the strains generated enterotoxin C as well as other types of enterotoxins. In Figure 6, columns 3 and 7 contain strains of Staphylococcus that produced none of the 5 most widespread types of enterotoxins. Columns 1, 2, 4 and 6, however, include the strains that produced enterotoxin A without being carriers of the entC gene. Both the ELISA kit and immunoblotting confirmed that the strains containing the entC gene produced enterotoxin.

**Discussion**

In this research, the existence of the entC gene in Staphylococcus strains and their ability to make enterotoxin were confirmed using both an ELISA kit with the sensitivity of RICASCREEN SET A, B, C, D, E (0.2 ng/ml) and immunoblot analysis (Figure 6).

![Figure 3](image-url) **Figure 3** The optimized conditions of the complete entC gene in Staphylococcus aureus strains. In picture A, wells 1-12 show the multiplied 1223bp fragment. Well 14 is the standard molecular weight of 1kb. Picture B shows wells 1-6 of preparing the PCR product for sequencing. Well 7 is the standard molecular weight of 1kb.
Figure 2) A comparison of the sequence provided by this study and the standard Staphylococcus aureus of the gene bank coded X.5815.1.
Figure 3) A comparison of the amino acid sequence obtained in this study with the translated sequence of the standard gene of entC1 reveals that, of amino acids 46-321, there is only one mismatch in amino acid 218.

Figure 4) The results of the ELISA reaction with the pre-provided in the kit of RICASCREEN SET A,B,C,C,E manufactured by the German company of r-Biofarm. The ELISA test was carried out following the instructions provided by the manufacturing company and the plate picture was scanned. This picture depicts the undersurface of the plate.
Only 11% of the entC-carrying strains produced enterotoxin C only, while other strains produced not only enterotoxin C but also other types of enterotoxins. The reason for this is not completely clear and it is not possible to say exactly why one bacterial strain can make more than one type of enterotoxin. The ability to produce multiple types of enterotoxins, however, has been reported by other researchers, too. As Ifesan et al showed in 2009, some Staphylococcus aureus strains generated more than one type of enterotoxin [26]. Many studies have been, or are being, carried out to gain a precise knowledge of the role of enterotoxins, of the mechanism of their functioning and of their specific receptors. Perhaps one of the reasons for these intensive researches on the role of enterotoxins of Staphylococcus aureus [27] is the provocation of various types of cytokines including IL-1 and TNF-α [28].

Since these poisons are involved in causing various diseases and detecting them is possible only by means of standard strains which cannot be bought and since the strains bought may not be compatible with the ecological features of this region, and because correct diagnosis can make for good treatment and can prevent later complications caused by these superantigenic enterotoxins, it is very important to develop a standard fast method for detection of enterotoxin-producing strains. Furthermore, the emergence, in recent years, of Staphylococcus bacteria which are resistant to methicillin and its relationship with the increased potential of toxigenicity have created worries [29, 30] because the variety of diseases caused by enterotoxins has made it necessary to develop new methods of detecting them and of treating related illnesses [31, 32]. Some countries have developed standard methods for detecting each and every strain which produces enterotoxins and have identified the accurate criteria for detecting such strains in food and clinical specimens. For instance, American Type Culture Collection (ATCC), which is a center for collecting and storing bacteria in the US, has developed standard methods for detecting all groups of bacteria. In a similar fashion, Marr et al (1993) studied the features of various types of enterotoxin C and reported the 240 amino acids for each of them. They showed that the sequence of the first 10 amino acids of their enterotoxins were ESQPDPPTPDE and the last 10 amino acids IEVHLTNG [33]. In fact, their gene sequence contains about 720 base pairs. Other researches have reported a fragment of 820 base pairs. There are yet other researches which have chosen parts of the toxin for detection purposes. For instance, some have developed a primer which multiplies a 102bp fragment or one that multiplies a 283bp fragment. Another study yet involves developing a primer which multiplies a 490bp fragment. As can be seen, none of the reported fragments can truly represent a type of enterotoxin C.

In this research, a primer was developed that multiplied a 1223bp fragment in order to trace and develop a standard method of detecting the entC gene. In fact, the part starting from the beginning of the peptide signal and 250bp after the gene was considered. In this way, the 1223bp fragment was multiplied. As shown in Figure 4, the bases 92-1057 were readable in the sequencing machine. Although a comparison of the results with the reference gene reveals mismatches in 4 bases only, the translated sequence demonstrated that the first 10 amino acids started from number 65 and continued up to number 304, and that there was a mismatch in one amino acid only. That is, as depicted in Figure 5, in number 208, alanine amino acid has replaced valine, which points to a new sequence which is local to this region.

Due to the importance of the pathogenicity of Staphylococcus aureus enterotoxins,
researches are continually done on standardizing the new methods of detecting them. For example, Fischer et al developed the quantitative method of real-time immuno-PCR using standard strains of the ATCC in order to detect enterotoxins A and B [34]. They were successful because of their access to the standard strains of the ATCC. Also, Veras et al (2008) detected enterotoxin C by developing a 283bp fragment. Since access to existing standard world strains is limited and geographical variations may affect the type of features attributed to the standard strains in other regions, identifying the necessary standards for detecting local strains of Staphylococcus aureus producing enterotoxins (including entC) is unavoidable. Since genetic variations have been reported in various strains of Staphylococcus aureus [35], using a reference gene model and by developing a proper primer, a method was developed for identifying the entC1-producing strains of Staphylococcus aureus. This is because relying on coagulase-positivity feature of the Staphylococcus bacteria in clinical specimens is not sufficient. As shown in Figure 6, in spite of the presence of the entC gene in them, the strains are different in terms of their ability to produce enterotoxin. Thus, the question is whether a bacterium which can produce more than one type of enterotoxin remains unchanged in terms of its level of pathogenicity and whether a fixed treatment would bring about a fixed result. In addition, some of the coagulase-negative strains isolated from clinical or food specimens produced various types of enterotoxins. Although different studies have reported different prevalence rates of enterotoxin-producing Staphylococcus aureus strains (5.3% to 88%), there is no precise and reliable statistics on the incidence rate of infections caused by such strains because there is no standard strain available. By standardizing a method of detecting strains producing enterotoxin C1, this study has partly prepared the situation for careful epidemiological studies of clinical specimens and food contaminations. Thus, standardized strains are ready to be offered to other researchers for further research.

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