Association of Staphylococcus aureus enterotoxin coding genes A, B and C and Atopic Dermatitis (Eczema)
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Abstract

Objectives: The present study was planned to detect the frequency of genes that is responsible for enterotoxin excretion in S. aureus isolated from dermal lesions of atopic dermatitis (AD) cases in AL-Najaf city, and to evaluate the single and combined existence of enterotoxins coding genes.

Methodology: A total of 54 Staphylococcus aureus isolates were applied to polymerase chain reaction specific for the amplification of various genes coding for Staphylococcal enterotoxins including 3 types (sea, seb and sec) with two sets each.

Results: As a result of DNA extraction and polymerase chain reaction directed to amplify the specific enterotoxin coding genes; The seb-6 gene was the most frequent gene , since it was detected in average of 8:36(22.22%), followed by the two sets of sec (sec-11 and sec-15) genes that comprised an equal average of 7:36(19.44%) each. Sea-2 gene set was detected in 6:36 (16.7%), while the lowest detection was seen for sea-1 and seb-7 since they were detected in 4:36(11.1%) each. Our results showed that single frequency was comprised 23:36 (63.9%), while the combined frequency was detected in 13:36(36.1%). The most frequent enterotoxin coding genes as a combined were sec-15 (19.44%), sea-2 (8.3%). Most single existence of replicated gene was detected for seb-6and sec-11 (19.44%) and (16.7%) respectively. Our results showed that absolutely single frequency was related to sea-1 gene (11.11%), while the absolutely combined frequency was seen in sec-15 (19.44%).

Conclusions: Polymerase chain reaction using newly designed primers is successful for identifying Staphylococcus aureus enterotoxins (A, B and C), seb-6 gene which was the most frequent.

Key words: Multiplex PCR, S. aureus, primers, atopic dermatitis


Introduction

Atopic dermatitis (AD) is a chronic inflammation of skin characterized with intense xerosis and pruritus. The pathogenesis of AD is a result of many factors including environmental, genetic, and immunological factors. The participation of Staphylococcus aureus in eczematous dermatitis affecting 10-20% of children and regarded as a major cause for morbidity, since patients with AD have a higher susceptibility for microbial colonization and an increased risk of skin infections [1,2]. There are more than 20 different staphylococcal and streptococcal exotoxins that may share sequence homology and are functionally related. These proteins are known with pyrogenic and are connected to different significant human diseases that include toxic shock syndrome and food poisoning [3]. Our study was planned for the detection of the frequency of genes (sea, seb and sec) that is responsible for enterotoxin excretion in S. aureus isolates by PCR method.

Material and Methods
DNA Extraction

All isolates were primarily examined by gram stain and by biochemical examinations, according to (Prescott, 2005). DNA Extraction has been achieved using Wizard®'s, genomic DNA purification kit (PRIMEGA, USA). The determination of DNA purity was done according to Sambrook et al. (1989)[4]. The purity and concentration of extracted staphylococcal DNA was determined by measuring the absorbance ratio at wavelength 260 nm over 280 nm using scandrop spectrophotometer (analyticajena-Germany). DNA sample was diluted with TE buffer solution to 1:10 and the optical density was read with spectrophotometer at wavelength 260nm and 280nm. The purity of DNA was measured by the equation of : $A_{260}/A_{280}=1.8-2.0$ (accepted range).

Preparation of the Primers Suspension

The DNA primers of different specific genes coding for various types of *S. aureus* enterotoxins were prepared by dissolving lyophilized product after centrifugation the mixture briefly with deionized DW (Bioneer, China), depending on the company's instructions and securities comment. It has diluted with deionized DW to give the stock solution, then the last Pico moles depending on company's instruction information were prepared.

Monoplex and Multiplex PCR Mixture

The genome that extracted from *S. aureus* isolates was subjected to PCR using primers mentioned in Table 1, by monoplex PCR. The protocol used depends on the manufacturer's instructions of Promega Co.: 12 μl of Master mix 2X, 1.5 μl from both forward (10μM) and reverse (10μM) primers, 5μl DNA template and 5μl PCR grade water to give a final volume of 25 μl. Up to four genes used in each run were used in multiplex PCR, by using the method listed by Rocchetti et al, 2018 [5], with the modification to be suitable for our study. The total volume of PCR tube content was 25μl; including 12 μl 2X Master mix, forward and reverse primers in size 0.5 μL plus 5 μL DNA from each isolate, and then completed the volume to 25 μL by DNA water.

Preparing the PCR

All positive controls were added into the PCR reaction together with the samples and the negative control (PCR-grade water). The component below was used for preparing the PCR. It is for only one reaction, These reagent values were multiplied with the sample number to find the values required for the master mix. About 12μl were Pipetted from the master mix into the PCR tubes, and 5μl of DNA (sample/positive or negative control) were added. The tube was then Close capped. Finally, Centrifugation was done at 14000rpm for about 30 sec.

PCR Thermo cycling Conditions

The PCR tubes were placed on the PCR machine and the right PCR cycling program parameters conditions (Optimase protocol writer™) were installed by using the link:


Table 1: : Primers that newly designed and suggested by the NCBI and IDT web sites, All these primers were *manufactured by Alpha DNA company, (Canada).
Programming the Sure cycler PCR Instrument

The thermal protocol for amplification of staphylococcal genes coding for various types of enterotoxins was applied. The PCR product was collected at the end step of the amplification cycle.

<table>
<thead>
<tr>
<th>Enterotoxin Type</th>
<th>Name</th>
<th>Oligo sequence (3'-5')</th>
<th>Product size (bp)</th>
<th>gene loci**</th>
<th>Synthesis No.</th>
<th>*Source of sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>sea-1</td>
<td>F/GAATTGCAGGGAACAGCTTTAG R/GTACCACCCGCACATTTGAT</td>
<td>251</td>
<td>121-143 352-372</td>
<td>562627 562628</td>
<td>Newly designed primers(NCBI)</td>
</tr>
<tr>
<td></td>
<td>sea-2</td>
<td>F/GGGAACAGCTTTAGGCAATCT R/ACTGTCTTGGACACCAATTTA</td>
<td>557</td>
<td>129-150 664-686</td>
<td>562629 562630</td>
<td>Newly designed primers(NCBI)</td>
</tr>
<tr>
<td>B</td>
<td>seb-6</td>
<td>F/CAGAGTCAACCAGATCTTCAA R/ATGCTCAGTTACACCACCCATAC</td>
<td>365</td>
<td>80-104 423-445</td>
<td>562637 562638</td>
<td>Newly designed primers(NCBI)</td>
</tr>
<tr>
<td></td>
<td>seb-7</td>
<td>F/ATGGTGCTGTTAATGGAGCATATAA R/GTGCGAGGACATCAGTCATC</td>
<td>268</td>
<td>425-447 673-693</td>
<td>562639 562640</td>
<td>Newly designed primers(NCBI)</td>
</tr>
<tr>
<td>C</td>
<td>sec-11</td>
<td>F/GGCAATCCTAGACCAGAAGCAA R/CTACACCCCGCATACATAC</td>
<td>344</td>
<td>60-85 388-408</td>
<td>562647 562648</td>
<td>Newly designed primers(NCBI)</td>
</tr>
<tr>
<td></td>
<td>sec-15</td>
<td>F/TCACTGGTCTAATGGATAATAG G/AGTTTGTCCTACAACAAATCTACG</td>
<td>216</td>
<td>104-130 297-320</td>
<td>562655 562656</td>
<td>Newly designed primers(NCBI)</td>
</tr>
</tbody>
</table>

Agarose Gel Electrophoresis and documentation

All requirements were prepared, technical applications of agarose gel electrophoresis for DNA visualization; detection and analysis were performed [6]. The amplified bands of PCR products were visualized by staining with ethidium bromide (0.5µg/ml) and photographed using Biometra gel documentation system. The positive results were recorded and distinguished when the DNA band base pairs of migrated sample equal to the specified target product size.

Assessment of DNA purity

The purity of DNA has been identified to Sambrook et al. (1989)[4]. Purity was determined by measuring the ratio of absorbance at 260 nm wavelength of 280 nm more using Spectrophotometer scandroid (analyticajena- Germany). It has been diluted with a sample of DNA solution with TE buffer solution to 1:10 and read the optical density with the spectrophotometer at a wavelength 260nm 280nm and. It was measured purity of DNA by the following equation: A260 / A280 = 1.8-2.0 (acceptable) range.

Statistical Analysis

Statistical analysis using mean ± SD, Chi-square test. In all tests, P <0.05 was considered statistically significant.
Results and discussion

Atopic dermatitis and enterotoxins coding genes

As a result of DNA extraction and polymerase chain reaction directed to amplify the specific enterotoxin coding genes; The seb-6 gene was the most frequent gene, since it was detected in average of 8:36(22.22%), followed by the two sets of sec (sec-11 and sec-15) genes that comprised an equal average of 7:36(19.44%) each. Sea-2 gene set was detected in 6:36 (16.7%), while the lowest detection was seen for sea-1 and seb-7 since they were detected in 4:36(11.1%) each (Figure 1) and Table 2. The frequency of enterotoxin coding genes was present in mean± SD of 6±1.67332.

Staphylococcus aureus isolated in our study that applied to PCR using specific primers were harboring the specific genes that in nature may produce super antigens, including types (SEA, SEB and SEC). This is in agreement with other reports [7]. This could affect the management of AD, since topical corticosteroids are the most common medication used for treatment of AD.

Table 2: The frequency of enterotoxins coding genes in SA isolated from a total of 54 Staphylococcus aureus isolates

<table>
<thead>
<tr>
<th>NO.</th>
<th>Gene</th>
<th>Gene name</th>
<th>Gene frequency</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SA enterotoxin A</td>
<td>sea-1</td>
<td>4</td>
<td>7.41%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sea-2</td>
<td>6</td>
<td>11.1%</td>
</tr>
<tr>
<td>2</td>
<td>SA enterotoxin B</td>
<td>seb-6</td>
<td>8</td>
<td>14.8%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>seb-7</td>
<td>4</td>
<td>7.41%</td>
</tr>
<tr>
<td>3</td>
<td>SA enterotoxin C</td>
<td>sec-11</td>
<td>7</td>
<td>12.9%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sec-15</td>
<td>7</td>
<td>12.9%</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>36:54</td>
<td>66.52:100</td>
</tr>
</tbody>
</table>

4-3: Single or combined enterotoxins coding genes in AD
Our results showed that single frequency was comprised 23:36 (63.9%), while the combined frequency was detected in 13:36 (36.11%). The most frequent enterotoxin coding genes as a combined were sec-15 (19.44%), sea-2 (8.3%). Most single existence of replicated gene was detected for seb-6 and sec-11 (19.44%) and (16.7%) respectively. Our results showed that absolutely single frequency was related to sea-1 gene (11.11%), while the absolutely combined frequency was seen in sec-15 (19.44%) (Table 3). $P<0.05$ was considered statistically significant. The frequency of singular and combined existence of enterotoxin coding genes was present in mean±SD of 3.83333±2.483277 and 2.16666±2.562551 respectively. Chi-square test revealed that there were no statistical differences at p value of less than 0.05.

**Table 3:** Single or combined frequency of enterotoxins coding genes in *S. aureus* isolates

<table>
<thead>
<tr>
<th>NO.</th>
<th>Gene</th>
<th>Gene name</th>
<th>Single frequency(%)</th>
<th>Combined frequency(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SA enterotoxin-A</td>
<td>sea-1</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sea-2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>SA enterotoxin-B</td>
<td>seb-6</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>seb-7</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>SA enterotoxin-C</td>
<td>sec-11</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sec-15</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>23:36(63.9%)</td>
<td>13:36(36.11%)</td>
</tr>
</tbody>
</table>

There are about 23 different SEs have been designated based on their antigenic characteristics (SEA to SE1Y) [8]. These enterotoxins may be classified as classical SEs (SEA, SEB, SEC, SED, and SEE) or as nonclassical SEs, including SEG, SHE, SEI, SES, SER, and SET, that exhibiting emetic activity [9]. Related toxins that not have emetic activity or have not been checked for emetic activity are similarly designated as staphylococcal enterotoxin-like toxins (SEls) [10,11], the fact that may be demonstrated the relatedness of samples that not corresponding to our genes.

Other reports also revealed the elevation in the enterotoxigenic isolates number as a consequence of the new SEs discovery, since 30% of the isolates had geneshat code for classic toxins with a frequency of 57% [12]. Multiple genes were also recorded in genetic profiles of *S. aureus* [13,14]. In Japan, Omoe et al. observed that 77.4% of the *S. aureus* isolates were positive for the presence of single and multiple genes that code for corresponding enterotoxins [15]. Our results about single or combined existence were shown in figures 1-6.

In other study, Nazari et al [14] showed that 28 (53.8%) isolates were produce at least one Se gene, in their work, six isolates (11.53%) were positive for genes encoding three enterotoxins. Multiple enterotoxin coding genes can be seen obviously in figure(2).

In the current study the SE genotypes isolated from healthy individuals carriers were also recorded that out of 10 isolates tested, 2 isolates (20%) were positive for SEA. These results are in agreement with previous finding of and Mehrotra et al., that healthy individuals may be carrier of toxin-producing strains of *S. aureus* [16]. However, the clinical and biological effects of these multiple toxins still under investigations since it are unclear whether all the toxins were with actually expression.

The results that recorded in this study can identify enterotoxin genes but does not ensure whether the gene is actually expressed or even whether that product encoded is really a functional protein. In another study done by Najera Sanchez et al. [17], the levels of association or correlation between genes presence that encode SE (by PCR application) and the same gene expression (by enzyme linked immunosorbent assay application) were showing 100% for SEA, 47% for SEB, 86% for SEC and SEE, 89% for SED, and . Thus, the actual expression of the toxin needs to be further assessed by activity or an immunological assay. As a demonstration for the multiple toxins presence in wide variety of SA strains is that these genes may be structurally linked as mentioned by Fitzgerald et al. [18].
The genes that encode for sel-like proteins, toxic shock syndrome toxin (tst) and other types of B,K, and SE are related to the pathogenicity islands of S. aureus isolates [19]. The pathogenicity involve the tst gene and an open reading frame (ORF) with similar sequence to those encoding SEs was also recorded by Lindsay et al., [20], and other regions include enterotoxin of types- D and type J [21]. In another study of 198 clinical isolates by Jarraud et al.,[22], 7 strains showed only a single enterotoxin gene of the sea gene, perhaps because it has been shown to be associated with a structurally unstable, possibly discrete, mobile genetic element that is regarded not part of the egc cluster.

Figure 2: The product of PCR electrophoresis picture: the gel constitutes of 1.5 % agarose with 0.5µg/ml Ethidium bromide staining and the electrophoresis was done under 100V for 1.5 hr. The picture was taken by gel documentation system (Biometra, UK). Lane1: Ladder of 1500bp,(lanes 9, 10 and 11 were presenting sea-2(557bp), sec-15 (216bp)and GSEAR(102bp)); (lanes 3,6, 7 and 12 were presenting sec-11(344bp,bp), Lan 8: presenting sec-11 and GSEAR; Lane 4,5: PCR product after using E.coli genome as a negative control. * The characteristics of gel,ethidium bromide concentration ,voltage and electrophoresis time were unified for all the following electrophoresis pictures. GSEAR: These data were excluded from our calculations in this research.

Figure 3: Showing the electrophoresis picture of the genome of Staphylococcus aureus isolates after PCR of sea-1(lan 2,6,8 and 9), sea-2(lan 17 and 18), seb-6 (lane 21,24,25 and 26); GSEAR (35,36 and 37); Lane10,19,20,29and 38: Ladder of 1500bp
Figure 4: Showing the electrophoresis picture of the genome of *Staphylococcus aureus* isolates after PCR of sec-11(lane 6); Lane1: Ladder of 1500bp.

Figure 5: Showing the electrophoresis picture of the genome of *Staphylococcus aureus* isolates after PCR of seb-6 gene (365bp) (lanes 1,5,10 and 14); Lane8: Ladder of 1500bp.

Figure 6: Showing the electrophoresis picture of the genome of *Staphylococcus aureus* isolates after PCR of seb-7 gene(lanes 1,2); Lane 3 presenting Sea-2 gene; Lane 9: Ladder of 1500bp.

References


