Detection of newly defined superantigenic toxin genes and coagulase gene polymorphism in *Staphylococcus aureus* isolates

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The current study aims to use coagulase (coa) polymorphism gene to identify *Staphylococcus aureus* isolated from stool samples, evaluate the efficiency of these methods in discriminating variable strains, and compare these subtypes with antibiotypes. A total of 100 specimens were collected from patients in Babylon province, Iraq, between July 2016 and September 2016. Twenty *S. aureus* strains were isolated and identified using standard laboratory microbiological tests. The bacterial isolates were then examined by coa gene restriction fragment length polymorphism genotyping. Out of 20 isolates, coa gene types were classified, and the amplification products showed multiple size bands (500, 600, 700, 800, and 900-bp bands). Coa gene PCR restriction fragment length polymorphisms exhibited seven patterns that ranged from one to four fragments with AluI digestion. The results have demonstrated that many variants of the coa gene are present. At least one type of *S. aureus* newly described enterotoxin gene (staphylococcal enterotoxins) was harboring in all 20 (100%) of the isolates. The most frequently encountered gene were sei (100.%), seh (5%), seg (65%). Many *S. aureus* isolates carry at least one of the enterotoxin genes, and (95%) strains harbored more than one toxin gene coding.

Introduction

*Staphylococcus aureus* is a very dangerous microorganism that can cause harmful diseases to humans and animals, such as endocarditic infection, septicemia, purulent inflammation, toxic shock syndrome, and food poisoning caused by staphylococcal super antigens [1,2].

*S. aureus* belongs a group of strong immunodeveloped toxins involved in the gastroenteritis and toxic shock syndrome. They are showing denaturation by heat and protein enzymes resistance. These toxins have the capacity to cross-link major histocompatibility complex (class II) molecules located on antigen-presenting cells with T-cell receptors. The production of the complex motivates an intense proliferation of T lymphocyte cells in an antigen-independent manner [3] causing production and releasing a heavy amount of immune stimulatory as cytokine that causes different symptoms such as leak off capillary, destruction epithelial cells, and hypotension. The primary role of these toxins as super-antigens is thought to debilitate immunity of the host to adequately allow the pathogen to disseminate and to advance. The staphylococcal super antigens A, B, C, D, E, G, and Q are accountable for food poisoning and toxic shock syndrome, by the *tst* gene that does coding for toxic shock syndrome toxin-1 [4].

Food poisoning is caused by *S. aureus* as it grows in huge numbers and generates extracellular toxins in food
products which may survive heating. This microorganism lives in different areas of the skins of humans and animals, and it is the common source of diseases, which can be transferred to food products [5].

*S. aureus* strains produced considerably different types of enterotoxins that have a scientific nomenclature (A, B, C, D, E, G, H, I, J, K, L, M, N, O, P, Q, R, and U). The first five (A to E) classical extracellular enterotoxins are known to cause 95% of the diseases such as food poisoning worldwide. These are dynamic, as more and more novel staphylococcal enterotoxins are being discovered [6].

There is a strong relation between the capacity of *S. aureus* strains to produce one or multiple of the enterotoxins and the existence of staphylococcal food poisoning [7]. These bacteria produce two types or forms of coagulase (coa) enzymes – the bound and free forms of coa, which is an enzyme that causes coagulation and clotting of blood in the human host [8].

The coa gene, coding for the coa enzyme, can be used for molecular diagnosis of *S. aureus*. The coa gene is greatly polymorphic due to variations in the sequence of the 3’ variable region. Many studied testings of the coa gene in a variety of staphylococcal strain have indicated a variety in the amino acid sequence and the number of tandem repeats at the 3’ end [9].

Epidemiologic typing of *S. aureus* strains by a number of molecular methods, such as amplification of the coa gene and PCR restriction fragment length polymorphism of the coa gene (PCR–RFLP), has been evolved [10]. Coa gene typing has been considered as a striking technique in clinical laboratories due to the ease of procedure and speed of its conducting [11]. Its discriminatory weight relies on the heterogeneous region that contains 81-base pairs tandem repeats at the 3’ end [12].

Expansions of these specific regions generate DNA fragments of various sized products that are huge in number and sequence polymorphisms, which concern these repeats of short tandem [13].

The current study aimed at using coa polymorphism gene to identify *S. aureus* isolated from stool samples, evaluate the efficiency of these methods in discriminating variable strains, and compare these subtypes with antibiotype.

**Materials and methods**

**Samples collection**

*S. aureus* isolates were identified according to standard biochemical tests [14]; then the identification was confirmed by using API system strips (Bionerieux, Marcy-l’Etoile, France). This study includes a total of 100 feces specimens from patients who were admitted to the main two hospitals in Al-Hilla City, Iraq (Hilla General Teaching Hospital and Merjan Medical City) during the period from July 2016 to September 2016. Stool specimens were collected from patients who were admitted to emergency room with specific symptoms as diarrhea and vomiting that are suspected of being infected by food poisoning according to their physicians. By using disposable, sterile, clean, leak-proof containers in a proper way to avoid any possible contamination, fecal specimen were diluted serially 10-fold in peptone water and cultured aerobically on culture media; the lowest measurement of detection was 400 CFU/g of feces. The different morphologies of colony were isolated, subcultured on other medium; gram stain was tested and finally cultured on blood agar, mannitol salt agar incubated at 37°C for 24 h for farther testing and bacterial diagnosis. The results indicate that the rate of *S. aureus* isolated from patient with gastroenteritis is 20%, other bacterial growth is 45%, and no bacterial growth is 35%.

**DNA extraction**

Whole bacterial DNA was extracted from *S. aureus* isolates by using Geneaid genomic DNA extraction kit (Bioneer, Alameda, California, USA) according to the manufacturer’s protocol. DNA electrophoresis was done in a 1% agarose gel, and primer sequences for (sei, seg, and seh) genes were designed according to reference [15].

**PCR amplification**

Bacterial DNA was used as a template for the finding of seg, seh, sei, and coa genes. A pair of specific primers used for the amplification of genes is shown in Table 1.

**Restriction fragment length polymorphism of coagulase gene**

An aliquot of 10-μl amplification product was digested with 2 U of restriction endonuclease *Alu* (Promega, Madison, Wisconsin, USA) at 37°C for 4 h. According to the kit, the resulted fragments were migrated on 2% agarose gels, which were stained with ethidium bromide, and the image was observed under ultra violet light. The protocols of RFLP mixture volumes are shown in Table 2.

**Results**

**Newly described enterotoxin genes (seh, sei, seg) detection**

Primers of *Staphylococcus*, a newly described enterotoxin gene, were used for the recognition of the presence of seh, sei, seg genes in *S. aureus*. It has been found that the percentages of detection were 5, 100, and 65%, respectively, with length of 360, 465, and 327 base pairs, when compared with allelic ladder (Figs 1–3).
Molecular genotyping of S. aureus by PCR–restriction fragment length polymorphism of coagulase gene

Coa genes were chosen due to their ubiquitous presence among S. aureus strain, and this gene proved to generate a very powerful typing method. Amplifications of coa gene from 20 isolate products in different types based on sizes ranging from 500 to 900 bp were recognized as coa types I (500 bp), coa type II (600 bp), coa type III (700 bp), coa type IV (800 bp), and coa type V (900 bp) (Fig. 4) and were found at 500 bp 5%, 600 bp 30%, 700 bp 50%, 800 bp 5%, and 900 bp 10%.

The majority of the isolates revealed coa type (III). The different sizes of coa gene and many RFLP patterns are shown in Fig. 5 and Table 3.

Discussion

In this study, the detection rate of seh gene in PCR was also closer to that obtained by the authors of [17,18] who have observed that the seh gene was presented in 7.7% of isolates. However, Fumihiko et al. [18] detected seh gene in a high rate (59.7%) of S. aureus isolates.

These observations suggest that S. aureus strains carrying seh can produce an adequate amount of staphylococcal enterotoxin H to cause toxicity. Therefore, staphylococcal enterotoxin H is suspected to be involved in Staphylococcus food poisoning.

Table 1. The primer sequences and PCR conditions.

| Genes | Primer sequence (5’-3’) | Size of product (bp) | PCR conditions | Reference |
|-------|-------------------------|----------------------|----------------|-----------|
| seg F | CGTCTCCACCTGTGAAGG      | 327                  | 94 °C for 1 min | [15]      |
| seg R | CCAATGATTTGCTATTCTG     |                      | 64–68 °C for 1 min 30× 72 °C for 5 min | [16]      |
| sei F | CAACTGAATTTCAACAGTAC    | 465                  | 94 °C 5 min, 95 °C 30 s | [15]      |
| sei R | CAGGCAAGTCACTCTCGT      |                      | 60 °C 1 min 40× 72 °C 45 s | [15]      |
| seh F | CAACTGCTGATTTAGCTAG     | 360                  | 94 °C 5 min 95 °C 30 s | [15]      |
| seh R | GTGAAATGAGTAATTCTAGG    |                      | 55 °C 45 s 35× 72 °C 1 min | [15]      |
| coa F | ATAGAGATGCTGACAGG       | Variable             | 94 °C 3 min, 94 °C 1 min | [15]      |
| coa R | GCTTCGATTGTGATG       |                      | 55 °C 1 min 30× 72 °C 1 min | [15]      |

Omoe et al. [19] observed that staphylococcal enterotoxin H was produced in significant quantity in a proper medium by an S. aureus strain carrying seh gene, but either small amounts of staphylococcal enterotoxin G and staphylococcal enterotoxin I or no detectable toxin was produced by S. aureus strains carrying seg and sei, respectively, and this may explain the low percentage of detection of this gene.

In this study, seg gene was found with the highest percentage. This result was also reported by Ghaleb et al. [20] who detected this gene in 64% of the enterotoxigenic strains of S. aureus. However, Omoe et al. [19] found that the rate of seg gene from isolates recovered from cases of food poisoning was 16.9%.

This difference in the prevalence of these genes among other researchers could be accountable to many factors such as geographical difference, which may be further influenced by the variant ecological origins of the isolated strains (food, humans, and animals), types of specimen and sample (blood, urine, stool, nasal swab, milk, and meat), number of samples, and methodology [20].

Omoe et al. [19] showed that sea, seg, and sei were most frequently detected simultaneously with other genes.

Table 2. Protocols of restriction fragment length polymorphism mixture volumes.

| No. | Contents of reaction mixtures | Volume (μl) |
|-----|-------------------------------|-------------|
| 1   | Sterile, deionized water      | 16.3        |
| 2   | RE 10× buffer                 | 2           |
| 3   | Acetylated BSA 10 μg/μl       | 0.2         |
| 4   | DNA template                  | 1           |
| 5   | RE 10 U/μl                    | 0.5         |
| Total volume | 20 μl                      |             |

RE, restriction enzyme.

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Fig. 1. Agarose gel electrophoresis at 70 V for 50 min for seg gene in Staphylococcus aureus. PCR product visualized under UV light at 280 nm after staining with ethidium bromide. L: Ladder with 2000 bp, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, were positive for this gene with amplicon size 327 bp. UV, ultra violet.
Also, the differences in the detection of staphylococcal toxin genes may occur due to other causes, such as geographical differences, which may be obtained from the variation in environmental conditions and the nature of the strains [21].

The differences in the rates of staphylococcal toxins present in that may occur due to the existence of multiple toxin genes in *S. aureus* is regarded rare.

The different sizes of amplicon product of the *coa* gene might be referred to as polymorphism between separate strains obtained from many sources. Other studies have also obtained accuracies in PCR product variation by using molecular technique of the *coa* gene analysis [22].

The *coa* gene and PCR–RFLP of the *coa* genotyping is an important method for infectious disease outbreak investigations in which the aim is meant to describe a local and temporal rise in the incidence of infection by a certain bacterial species. The typing of outbreak strains eases the development of outbreak control strategies, defining the degree of the epidemic spread of bacterial strains and the number of clones involved in the transmission and infection, observation of the reservoirs of epidemic clones, and control the evaluations of the efficacy of control
Type I 700 700 10
Type II 220–240–700 700 5
Type III 80–220–240 600 20
Type IV 80–240–500 800 20
Type V 200–300 700 25
Type VI 80–220 900 5

Coa, coagulase; RFLP, restriction fragment length polymorphism.

Conclusion

Genotyping based on the RFLP of coa gene has been regarded as a simple and exact technique for printing of S. aureus isolated from different sources. The coa gene is genetically changeable. This polymorphism exists because of multiallelic types on the 3' end of the gene that differ in their sequence, and variation in size of products refers to coa gene. Also, the polymorphisms exist in coa gene in various S. aureus isolates; the restriction digestion of the amplicons of coa gene gave different restriction patterns regarding the newly detected seg, seh, and sei genes with the classic sea and seb genes; the frequency of potentially enterotoxigenic S. aureus would considerably be increased among the isolates from 15 to 45%.

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Conflicts of interest

There are no conflicts of interest.

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