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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 *Alu*I 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.

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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

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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 studded 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′ ends [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 antibiotypes.

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 (Biomerieux, 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*I (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).

Table 1. The primer sequences and PCR conditions.

Genes	Primer sequence (5′-3′)	Size of product (bp)	PCR conditions	Reference
seg F	CGTCTCCACCTGTTGAAGG	327	94 °C for 1 min	[15]
seg R	CCAAGTGATTGTCTATTGTCG		64–68 °C for 1 min 30×	
			72 °C for 5 min, 72 °C for 5 min	
sei F	CAACTCGAATTTTCAACAGGTAC	465	94 °C 5 min, 95 °C 30 s	[15]
sei R	CAGGCAGTCCATCTCCTG		60 °C 1 min 40×	
			72 °C 45 s	
seh F	CAACTGCTGATTTAGCTCAG	360	94 °C 5 min 95 °C 30 s	[15]
seh R	GTCGAATGAGTAATCTCTAGG		55 °C 45 s 35×	
			72 °C 1 min	
coa F	ATAGAGATGCTGGTACAGG	Variable	94 °C 3 min, 94 °C 1 min	[16]
coa R	GCTTCCGATTGTTCGATGC		55 °C 1 min 30×	
			72 °C 1 min	

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 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.

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

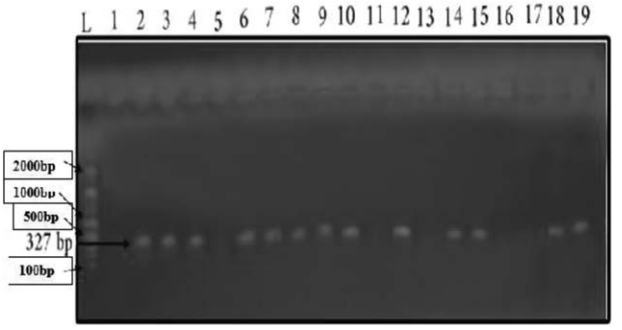

Fig. 1. Agarose gel electrophoresis at 70 V for 50 min for *seg* gene in *Staphylococcus aureus*. PCR product visualized under UV light at 280 nm after staining with ethidium bromide. L: Ladder with 2000 bp. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, were positive for this gene with amplicon size 327 bp. UV, ultra violet.

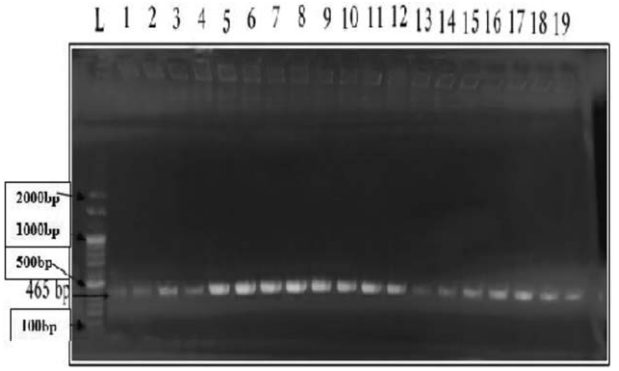

Fig. 2. Agarose gel electrophoresis at 70 V for 50 min for *sei* gene in *Staphylococcus aureus*. PCR product visualized under UV light at 280 nm after staining with ethidium bromide. L: Ladder with 2000 bp. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 were positive for this gene with amplicon size 456 bp. UV, ultra violet.

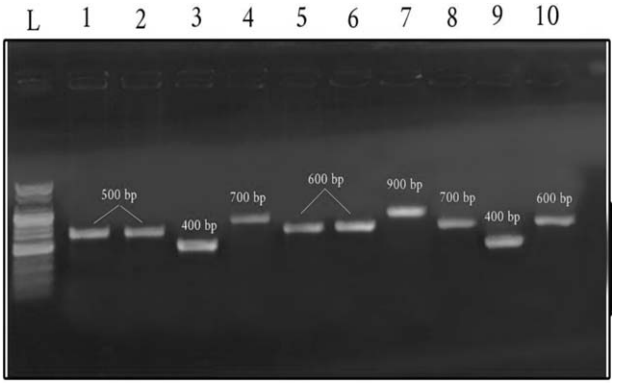

Fig. 4. Agarose gel electrophoresis at 70 V for 50 min for *coa* gene in *Staphylococcus aureus*. PCR product visualized under UV light at 280 nm after staining with ethidium bromide. L: Ladder with 2000 bp. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, were positive for this gene with amplicon size 400–900 bp. UV, ultra violet.

in the isolates that were derived from food poisoning flare-up.

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

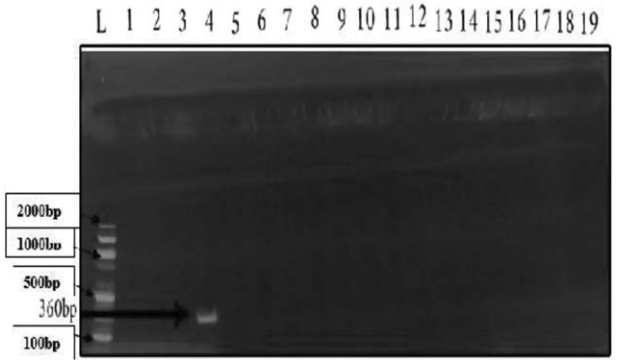

Fig. 3. Agarose gel electrophoresis at 70 V for 50 min for *seh* gene in *Staphylococcus aureus*. PCR product visualized under UV light at 280 nm after staining with ethidium bromide. L: Ladder with 2000 bp. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 were positive for this gene with amplicon size 360 bp. UV, ultra violet.

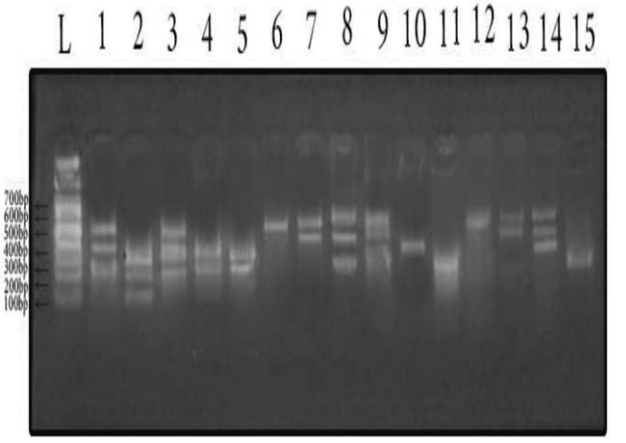

Fig. 5. Agarose gel electrophoresis at 70 V for 50 min for *AuI* I restriction fragment *coa* gen in *Staphylococcus aureus*. PCR product visualized under UV light at 320 nm after staining with ethidium bromide. 2% Agarose. L: Ladder with 2000 bp. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 with restrict of different size and type I, type II, type III, type IV, type IX, type X, type XI. UV, ultra violet.

Table 3. Restriction fragment length polymorphism patterns of coagulase gene.

No.	PCR–RFLP typing	Size of *Alu I* fragments (bp)	Coa PCR product (bp)	No. of isolates (%)
1	Type I	700	700	10
2	Type II	220–240–700	700	5
3	Type II	600	600	15
4	Type IV	80–240–500	800	20
5	Type IX	80–220–240	600	20
6	Type X	200–300	700	25
7	Type XI	80–220	900	5

Coa, coagulase; RFLP, restriction fragment length polymorphism.

plans, such as monitored of vaccination process efficacy [22].

The *coa* gene is polymorphic and molecularly changeable. This polymorphism exists due to multiallelic types on the 3′ end of the gene that differ in their arrangement. The variation in the size of the products of *coa* genes comprises three distinguished sites: the N-terminal of prothrombin-binding site, a central highly conserved region, and a C-terminal region comprising 5, 6, 7, and 8 U of the 81-tandem repeat [13].

The *Aul* I restriction enzyme cutting of the amplicon *coa* product generated different *Aul* I restriction patterns with the number of fragments varying from one to three and with the size of the fragment varying from 80 to 700 bp as shown in Fig. 5 and Table 3.

The results of PCR for the amplifications of *coa* gene in this study were also reported by several authors who revealed that PCR for gene amplifications of *coa* show profound polymorphism with preponderance of single or more amplified products of *coa* gene [23].

The genotypic difference was noticed as mutation in the sequence of codon or number of plasmids [24]. Virulence factors' variety and their combinations might produce an alteration in the level of diseases and dissemination of infections within and among animals [24].

Also, the results refer to differences in the *coa* gene of bacterial strains. These results were in concordance with the results obtained in several studies [22,25] concerning other strains of these bacteria. The variation among RFLP of the *coa* gene may occur because of an alteration in the sequence of the *coa* gene among different isolates that give rise to different restriction location [26].

The results in this study also revealed that some strains (20%) were not digested by *Aul* I enzyme; this might happen because of the loss of the restriction site of the enzyme in variable regions of the gene in these isolates that could result due to the point mutation in the repeated region of the *coa* gene [27].

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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