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Phylogenetic Relationships among Enterotoxigenic Clinical *Staphylococcus aureus* Isolates

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Abstract: A total of 50 *Staphylococcus aureus* strains were isolated from patients in Ain Shams University Hospital in Cairo. The presence of Staphylococcal Enterotoxin (SE) genes (*sea*. *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei* and *sej*) and the correlation of their prevalence with the genotypes were studied in these isolates. Polymerase Chain Reaction (PCR) of SE genes indicated that 36% of the isolates were enterotoxigenic. The prevalence of *sea* and *seb* plus *sec* among the total clinical isolates was 22 and 2%. Sixteen percent of the total isolates were *seg* positive, whereas 12%, 2% and 2% were *sei*, *she* and *sej* positive, respectively. All isolates containing *sei* were positive for *seg*, whereas none of the isolates harboured *sed* or *see* genes. Isolates were characterized by molecular biology tools, viz., randomly amplified polymorphic DNA (RAPD), PCR-RFLP of 16S rDNA and nucleotide sequencing for *sea* gene. The RAPD was used to test which isolates harbouring the toxin genes were genetically clustered. A total of 7 genotypes were identified at a 65% similarity level. Genotypes III accounted for the largest number of enterotoxigenic isolates (12 %), while genotypes IV and VII included a great diversity of enterotoxigenic isolates (*sea*, *seb*, *sec*, *seg*, *she*, *sei* and *sej*). Genotyping by PCR-restriction fragment length polymorphism analysis of the 16S rDNA gene revealed that 40% of the isolates were belong to type A, 32% to type B and 28% to type C. Most and great diversity of enterotoxigenic isolates belonged to genotype B. This study has demonstrated that the *sea* was the most dominant enterotoxin followed by *seg* and *sei* genes. The presence of the enterotoxin genes was independent ($P<0.05$) of the genotypes of the tested *S. aureus* isolates and the presence of the toxin genes is not genotype specific. Further more, *sea* gene sequence of the isolates being tested showed nucleotide variations at multiple sites when compared with other sequences available in the database.

Key words: Staphylococcal enterotoxin · RAPD · RFLP · restriction fragment length polymorphism

INTRODUCTION

Staphylococcus aureus causes multiple diseases in man and animals. Clinical isolates of *S. aureus* produces a variety of extracellular toxins and virulence factors that contribute in its pathogenic potential [1]. Enterotoxin production has been associated with clinical isolates in patients suffering from toxic shock syndrome, staphylococcal scarlet fever, recalcitrant erythematous desquamating disorder and arthritis [1-4]. Staphylococcal Enterotoxins (SE) are a group of single-chain, low-molecular weight proteins (molecular weight, 26900-29 600 Da) that are similar in composition and biological activity but differ in antigenicity [5]. The SEs are classified by serological criteria into five major groups: SEA, SEB, SEC (can be further subdivided into SEC1, SEC2, SEC3 based on differences in minor epitopes), SED and SEE [6,7]. Recently, other SEs identified as SEG, SEH, SEI, SEJ, SEK, SEL, SEM, SEN, SEO, SEP, SER and SEU [8-10].

The methods most frequently used for the detection of staphylococcal toxins are immunodiffusion, agglutination, radioimmunoassay, enzyme-linked immunosorbent assay and Polymerase Chain Reaction [11]. The enterotoxin genes are not distributed uniformly among *S. aureus* strains and the prevalence of these genes have been reported in different countries by many investigators [7, 12-14]. The production of toxins can vary significantly with staphylococcal genotype and genetic variation among these strains occurs in both enterotoxin and core genes [7, 15, 16]. Therefore, the molecular typing of *S. aureus* has proved useful for epidemiological

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studies, by determination of strain origin, clonal relationships between isolates and epidemiology of outbreaks [15-18]. These genotyping methods include pulsed-field gel electrophoresis [19, 20], multilocus sequencing typing [21, 22], random amplification of polymorphic DNA (RAPD) [17, 23] and PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the protein A gene (*spa*) [24], coagulase gene [25], 16S rRNA [26] and *aroA* gene [27].

Random amplified polymorphic DNA (RAPD) analysis is a technique following the first approach for rapid detection of genomic DNA [28, 29], utilizing a single short oligonucleotide primer of arbitrary sequences in a PCR, which has been found to be a simple, rapid and effective method for genotyping of *S. aureus* [30]. The PCR-RFLP analysis of the 16S rDNA gene will help in quick, easy and reasonable investigation the phylogenetic relationships of *S. aureus* strains [26, 31]. Also the DNA sequence of a target gene is one of the most promising for detection of genomic and somatic mutations, identification of strains and detection of allelic imbalances.

This study was designed to: (i) detect the presence of the five classical enterotoxin genes (*sea*, *seb*, *sec*, *sed* and *see*) and new enterotoxin genes (*seg*, *seh*, *sei* and *sej*) in Egyptian clinical isolates of *S. aureus*, (ii) investigate the genetic variation among these isolates by using RAPD and PCR-RFLP of 16S rDNA, (iii) determine the genotype specificity of the classical and newly described enterotoxin genes and (iv) describe the sequence variation in some regions of *sea* gene.

MATERIALS AND METHODS

Samples collection and identification: Fifty clinical isolates of *Staphylococcus aureus* were collected, identified by biochemical tests and determined the susceptibility of isolates to methicillin and various antibiotics during a previous study [32]. These isolates were obtained from various clinical specimens submitted to Ain Shams University Hospital in Cairo, Egypt.

Preparation of genomic DNA: Genomic DNA was extracted from overnight cultures of *S. aureus* using the Wizard Genomic DNA Purification kit (Promega). The procedure was identical to that recommended by the manufacturer. The preparations were analyzed on a 0.7% agarose gel and the quantity and quality of DNA were determined spectrophotometrically [33]. The amount of DNA was adjusted to the required concentration for genotyping of *S. aureus* isolates.

Detection of enterotoxin genes by PCR

Detection of *sea*, *seb*, *sec*, *sed* and *see* genes: Genomic DNA (50ng) of *S. aureus* strains was amplified in two sets of multiplex PCR as reported by Rosec and Gigaud [9]. Set A contained 3 ng/µl of each *sea, sed* and *see* primer, while set B contained 3 ng/µl of each seb and sec primer. Amplification with these primers gave rise to PCR products of 544, 416, 257, 334 and 170 bp for *sea*, *seb*, *sec*, *sed* and *see*, respectively. The PCR products were detected in 2% agarose gel in presence of the DNA marker and photographed according to Rosec and Gigaud [9].

Detection of *seg*, *seh*, *sei* and *sej* genes: Genomic DNA (50ng) of *S. aureus* strains were amplified by PCR using 3 ng/µl of each primer specific for *seg, seh, sei* and *sej* genes [9]. DNA amplification was carried out in a Perkin-Elmer thermocycler according to the procedure of Rosec and Gigaud [9]. The amplification with the specific primers gives rise to PCR products of 400, 357, 467 and 426 bp for *seg*, *seh*, *sei* and *sej*, respectively. The PCR products were analyzed by 2% agarose gel electrophoresis in presence of the DNA marker.

Genotyping of *S. aureus*

RAPD-PCR typing: Three random-sequence primers were used in three separate RAPD-PCR tests for typing of S. aureus isolates. Primers R2 (5'GAGCCAGCGTCCATCGGCCACCA-3') and OPA14 (5'-GACCGCTTGT-3') and primer OPA13 (5'-CAGCACCCAC-3') [Operon Technologies, Inc., Atlanta, USA] were selected because they previously showed good discriminatory power in RAPD analysis of S. aureus [34]. RAPD-PCR was carried out in a 25 µl reaction mixture containing 2.5 µl 10x buffer, 0.2 mM dNT'Ps, 100 pmol primers, 2 U Taq DNA polymerase, 3. mM MgCl2, 50 ng DNA template and nuclease-free water. Amplification conditions consisted of denaturation at 95°C for 5 min and 40 cycles of denaturation at 95°C for 1 min, annealing at 33°C for 1 min and extension at 72°C for 1 min with a final extension at 72°C for 10 min. PCR products were detected in 2% agarose gel.

Cluster analysis of RAPD assay: RAPD banding patterns of the 50 isolates of *S. aureus* were examined and the bands were scored, with the data coded as a factor of 1 or 0, representing the presence or absence of bands, respectively. Cluster analysis was used to

produce dendrogram showing estimates of the distance values and to analyze the genetic relatedness among 50 strains of *S. aureus*. Thirty-two bands of the summed results for the three primers were used for cluster analysis in RAPD assay. The dendrogram based on the similarities was derived from the unweighted pair group method using arithmetic averages (UPGMA).

PCR-RFLP analysis of the 16S rRNA gene: PCR amplification of *S. aureus* 16s rRNA gene was performed as following: 2.5 µl 10x buffer, 0.2 mM dNT'Ps, 2 U Taq DNA polymerase, 2.5 mM MgCl2, 50 ng DNA template, 1µl (0.3 µg/ml) of each RW01 primer (5'-AAC TGG AGG AAG GTG GGG AT-3') and DG74 primer (5'-AGG AGGTGA TCC AAC CGC A-3'), the volume was made up to 25µl with 2.5µl of nuclease free water. DNA amplification was carried out in a Perkin-Elmer thermocycler, with the following thermal cycling profile: initial denaturation at 94°C for 4 min was followed by 35 cycles of amplification (denaturation at 94°C for 45 s, annealing at 58°C for 45 s and extension at 72°C for 60 s) ending with a final extension at 72°C for 2 min. The PCR products were detected in 3% agarose gel in presence of the DNA marker and photographed according to Ghassan *et al.* [35] with some modification. The RFLP assay was carried out by individual digesting the PCR products with Taq1, RsaI, HindIII, AluI and MspI enzymes (Promega, USA), then the products were analyzed by 3% agarose gel electrophoresis.

DNA sequencing and phylogenetic construction: DNA sequence for the enterotoxin A (*sea*) gene was performed by Macrogene company (Korea). Three isolates, one from blood, one from sputum and one from pus were selected as representative isolates for enterotoxin A gene sequencing for detecting intraspecific polymorphism.

All the sequences were submitted to NCBI GenBank database and the accession numbers were shown in table 3. DNA sequences were aligned in ClustaW program. The Neighbouring-joining tree was established in MEGA 3.1 program [36] using Tamura and Nei genetic distance method [37]. The sequenced *sea* genes were alignment compared with four different *sea* S. *aureus* available in the GenBank database.

Statistical analysis: X^2 test was used to study the correlation between the prevalence of the enterotoxin genes and the genotypes of *S. aureus* isolates. A value of $P<0.05$ was considered statistically significant [38].

RESULTS

Prevalence of the classical enterotoxin genes *sea*, *seb*, *sec*, *sed* and *see*: A total of 12 (24%) clinical isolates of *S. aureus* were harbouring *sea*, *seb* and *sec* genes, either singly or in combination. Eleven of the total isolates were positive for the *sea* gene and one was positive for both *seb* and *sec* genes. None of the isolates harboured *sed* or *see* genes (Table 1). The sizes of the amplicons obtained for the enterotoxin genes corresponded to the anticipated sizes reported by Rosec and Gigaud [9] (Fig. 1).

Prevalence of enterotoxin genes *seg*, *seh*, *sei* and *sej*: A total of 16 (32%) of the tested *S. aureus* isolates were positive for the newly described enterotoxin genes, with the presence of one or more of the *seg*, *seh* and *sei* genes per isolate. Eight (16%) of the total isolates were *seg* positive, six (12%) were *sei* positive, one (2%) was *seh* positive and one (2%) was positive for the *sej* gene (Table.1). All the isolates that contained the *sei* gene 6 (12%) also had *seg* gene and the positive *seh* gene isolate was also positive for *seg*.

The presence of the classical SE genes was observed simultaneously with the presence of the newly described SE genes in 3 (6%) out of 50 tested strains, while eight (16%) strains harbored classical SE gene(s) only (Table 2 and Fig. 3).

RAPD analysis: RAPD assay of 50 *S. aureus* isolates was performed three primers showed consistently different banding patterns with reproducible polymorphic bands of variable size and number. Amplification with primer R2 generated 4 monomorphic bands and 8 polymorphic

Table 1: Distribution of enterotoxin genes production in clinical *S. aureus* isolates according to the source of the isolates

Type of enterotoxin genes	No. of positive isolates (%)*	Source of clinical isolates (%)				
		Blood	Wound	Sputum	Pus	Urine
sea	11 (22)	3 (6)	2 (4)	3 (6)	3 (6)	0 (0)
seb	1 (2)	0 (0)	1 (2)	0 (0)	0 (0)	0 (0)
sec	1 (2)	0 (0)	1 (2)	0 (0)	0 (0)	0 (0)
sed	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
see	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
seg	8 (16)	1 (2)	3 (6)	3 (6)	1 (2)	0 (0)
seh	1 (2)	0 (0)	1 (2)	0 (0)	0 (0)	0 (0)
sei	6 (12)	0 (0)	2 (4)	3 (6)	1 (2)	0 (0)
sej	1 (2)	0 (0)	0 (0)	0 (0)	1 (2)	0 (0)
Total	29 (58)	4 (8)	10 (20)	9 (18)	6 (12)	0 (0)

*Some isolates carried more than one SE genes

Table 2: Distribution of enterotoxin genes among Egyptian *S. aureus* genotypes

No. of isolates	Type of enterotoxin genes	Source of clinical isolates	Genotype	
			RAPD	RFLP
22	*sea*	Blood	I	B
5	*sea*	Wound	II	B
39	*sea*	Blood	III	B
38	*sea*	Sputum	III	B
7	*seg, sei*	Sputum	III	B
15	*sea*	Pus	III	B
9	*sea*	Blood	III	B
4	*sea*	Sputum	III	B
26	*seb, sec, seg, sei*	Wound	IV	B
10	*sej*	Pus	IV	B
48	*sea, seg, sei*	Wound	V	C
29	*sea*	Pus	V	C
16	*sea*	Pus	V	C
44	*seg, sei*	Sputum	VI	C
50	*seg*	Blood	VI	C
20	*sea, seg, sei*	Sputum	VII	A
47	*seg, seh*	Wound	VII	A
12	*seg, sei*	Pus	VII	A

Table 3: Reference and examined species sequenced for *sea* gene of *S. aureus*

Gene	Source of strain	Genotype		Accession number
		RAPD	RFLP	
sea	Blood	III	B	EF614245
Sea	Pus	V	C	EF614246
sea	Sputum	VII	A	EF614247

bands in total 12 RAPD patterns. Primer OPA 14 produced 3 monomorphic bands and 8 polymorphic bands in total 11 RAPD patterns and OPA13 primer generated 4 monomorphic bands and 5 polymorphic bands in total 9 RAPD patterns (Fig. 2). Actually a total of 32 distinct bands were obtained and used for cluster analysis.

Figure 3 shows a dendrogram constructed on the basis of similarity index among *S. aureus* isolates using the three RAPD primers. A 65% similarity cut-off value gave 7 major clusters (RAPD genotypes) (I-VII). The majority of *S. aureus* isolates (18) belonged to genotype VII. The others isolates were distributed as follows: 9 isolates in genotype VI, 7 isolates in genotype V, 3 isolates in genotype IV, 9 isolates in genotype III, two isolates in genotype II and two isolates in genotype I.

The distribution of the enterotoxin genes among *S. aureus* genotypes is shown in Table 2 and Fig. 3. The *sea* gene was carried by some isolates of genotypes I, II, III, V and VII. The *seb* and *sec* genes were carried in combination by one isolate belong to genotype IV. The *seg* gene was carried either singly or in combination with the *sei* gene by some isolates of genotypes III, IV, V, VI and VII. On the other hand, *seh* was present in one isolate belong to genotype VII and *sej* was present in one isolate belong to genotype IV. The finding that some SE positive and some SE negative strains generated identical or similar amplicon-profiles suggests that SE positive strains do not belong to a specific genetic class. Also, X^2 test showed that the overall presence of the enterotoxin genes was independent of genotype. There was no significant difference ($P<0.05$) in the prevalence of the tested SE genes in the different *S. aureus* genotypes.

PCR-RFLP analysis of 16S rDNA gene: PCR products of 16S rDNA for all S. aureus were digested with Taq1, RsaI, HindIII, AluI and MspI. No cutting activity with; AluI and MspI were observed. In addition, RsaI and Taq1 showed similar profiles for all strains. On the other hand, HindIII produced obvious discrimination between the tested isolates as shown in Fig. 4. HindIII enzyme produced three different profiles which reflected the all tested strains were divided into three types A, B and C. 16S rDNA gene RFLP pattern 1(type A) was most common (20 [40%] of the isolates/strains examined) (Fig. 4, lane 2), followed by 16S rDNA gene RFLP pattern 2 (16 [32%] isolates) (type B) (Fig. 4, lane3) and RFLP pattern 3 (14 [28%] isolates) (type C) (Fig. 4, lane 4).

Figure 5 is a scheme that shows the majority of enterotoxigenic *S. aureus* isolates (10) belonged to genotype B. The *sea* gene was carried by 7 isolates in genotype B, 3 isolates in genotype C and one isolate in genotype A. The *seb* and *sec* genes were carried in combination by one isolate belong to genotype B. The *seg* gene was present singly or in combination with the *sei* gene in all genotypes as follows: 3 isolates in genotype A, 3 isolates in genotype C and 2 isolates in genotype B harbored *seg* gene. Whereas *sei* gene was carried by two isolates in all genotypes (A, B and C). On the other hand, *seh* was present in one isolate belong to genotype A and *sej* was present in one isolate belong to genotype B. The presence of the enterotoxin genes was independent ($P<0.05$) of the genotypes obtained by PCR-RFLP of 16S rDNA gene of the tested *S. aureus* isolates.

***sea* sequence analysis:** Sequencing for staphylococcal enterotoxin A (*sea*) genes of three strains which isolated from different sources (blood, sputum and pus) was performed and the sequence alignment was carried out for these isolates using ClustaW. More than ten nucleotide variations were observed in the *sea* gene sequence of

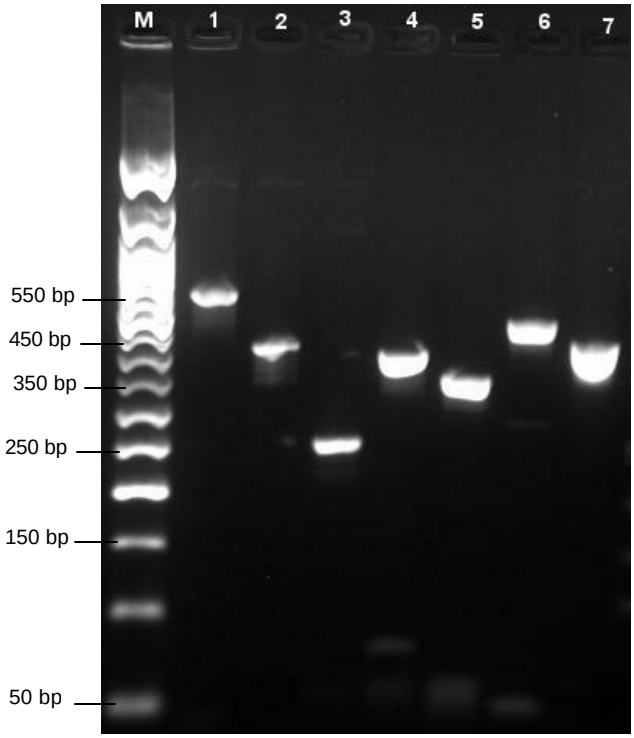

Fig. 1: Agarose gel electrophoresis showing specific PCR amplification products for *S. aureus* enterotoxin genes. Lanes: M, DNA molecular size marker (50 bp ladder; Promega); 1, *sea*-positive Isolate (544 bp); 2, *seb*-positive Isolate (416 bp); 3, *sec*-positive Isolate (257 bp); 4, *seg*-positive isolate (400 bp); 5, *seh*-positive isolate (357 bp); 6, *sei*-positive isolate (467 bp); 7, *sej*-positive isolate (426 bp)

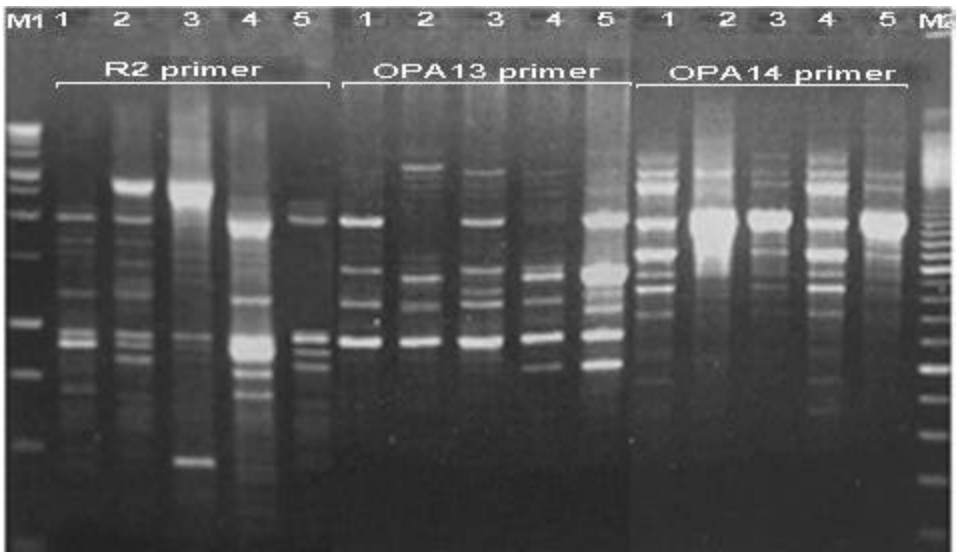

Fig. 2: Representative 2% agarose gels of RAPD-PCR patterns generated from three primers using: R2, OPA13 and OPA14; lanes 1–5, *S. aureus* isolates were representative for 50 tested isolates; M1, DNA molecular size marker (1 kb ladder; Promega); M2, DNA molecular size marker (100 bp ladder; Promega)

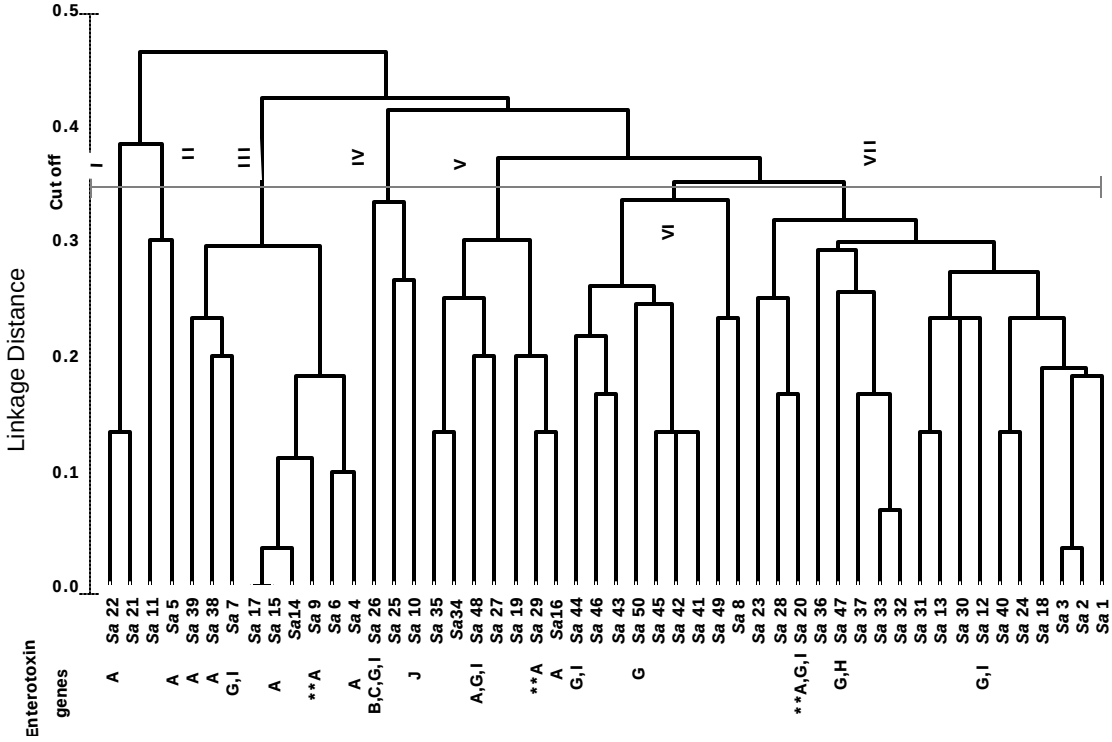

Fig. 3: RAPD-based dendrogram showing genetic relatedness and distribution of enterotoxin genes among *S. aureus* isolates

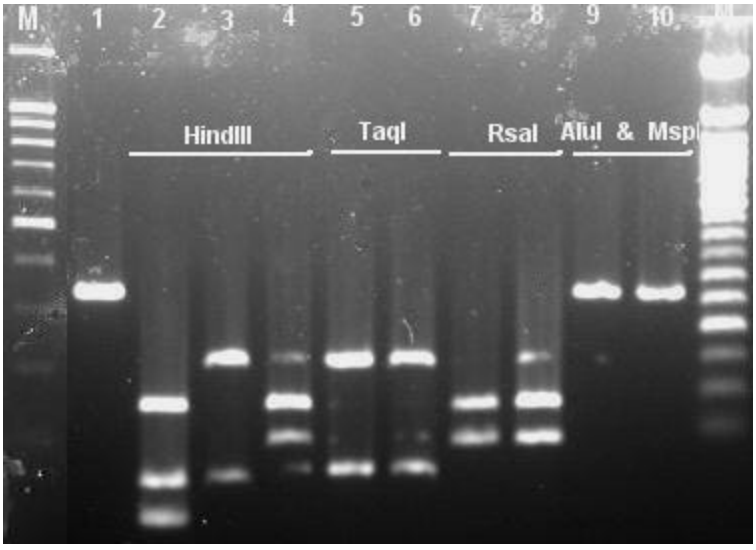

Fig. 4: RFLP pattern for the 16S rDNA gene of *S. aureus.* Lane: 1, 370-bp PCR amplification product of 16S rDNA gene; Lanes 2-4, HindIII digestion type A, type B, and type C, respectively; Lanes 5-6, TaqI digestion; Lanes 7-8, RsaI digestion; Lanes 9-10, undigested 16S rDNA PCR products; Lanes: M, DNA molecular size marker (GeneRuler™ 50 pb DNA ladder, MBI Fermentans)

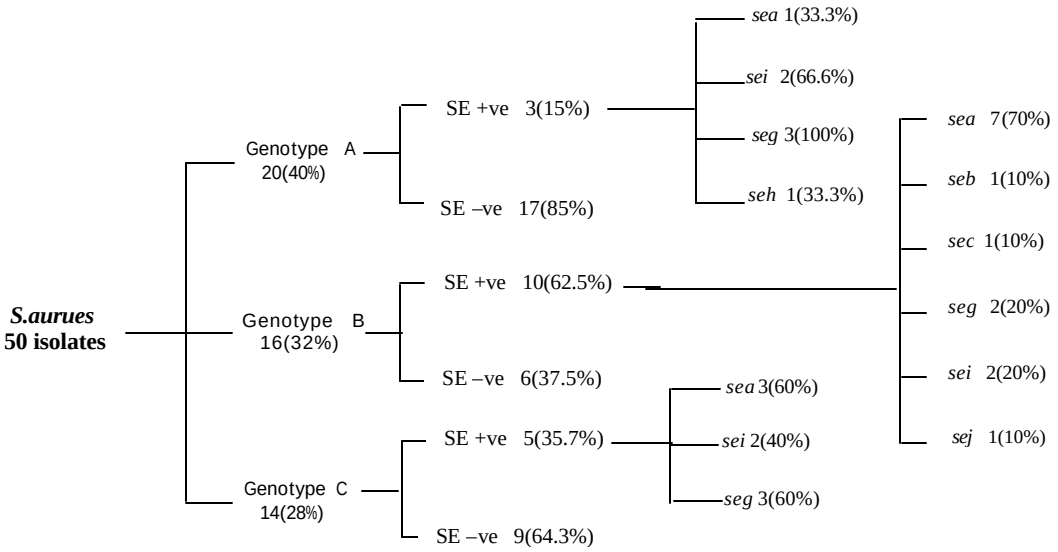

Fig. 5: Scheme representing the relationship between the genotypes of *S. aureus* and the presence of the enterotoxin genes, where the number in parenthesis indicates the percentage in respect to the former number in the scheme. ⁻ve = negative, +ve = positive

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EF614245          GATTAGGATTTTAA--TATTCATGG-ATA-CGATTTATTAGTAGATTTTGATTCAAAG 54
EF614246          TGTAGGTATTTTAAGACATTCGTGGTATAACGATTTATTAGTAGATTTTGATTCAAAG 60
EF614247          GTGTAGCTTTTTAG-ATATTCGTGGTATA-CGATTTATTAGTAGATTTTGATTCAAAG 57
                        *  *****    **** *** *** ****************************

EF614245        GATATTGTTGATAAATATAAAGGGAAAAAAGTAGACTTATATGGTGCTTATTATGGTTAT 114
EF614246        GATATTGTTGATAAATATAAAGGGAAAAAAGTAGACTTGTATGGTGCTTATTATGGTTAT 120
EF614247        GATATTGTTGATAAATATAAAGGGAAAAAAGTAGACTTGTATGGTGCTTATTATGGTTAT 117
                ************************************* ********************

EF614245        CAATGTGCGGGTGGTACACCAAACAAAACAGCTTGCATGTATGGTGGTGTAACGTTACAT 174
EF614246        CAATGTGCGGGTGGTACACCAAACAAAACAGCTTGTATGTATGGTGGTGTAACGTTACAT 180
EF614247        CAATGTGCGGGTGGTACACCAAACAAAACAGCTTGTATGTATGGTGGTGTAACGTTACAT 177
                ********************************** ************************

EF614245        GATAATAATCGATTGACCGAAGAGAAAAAAGTGCCAATCAATTTATGGCTAGACGGTAAA 234
EF614246        GATAATAATCGATTGACCGAAGAGAAAAAAGTGCCGATCAATTTATGGCTAGACGGTAAA 240
EF614247        GATAATAATCGATTGACCGAAGAGAAAAAAGTGCCGATCAATTTATGGCTAGACGGTAAA 237
                ********************************** ************************

EF614245        CAAAATACAGTACCTTTGGAAACGGTTAAAACGAATAAGAAAAATGTAACTGTTCAGGAG 294
EF614246        CAAAATACAGTACCTTTGGAAACGGTTAAAACGAATAAGAAAAATGTAACTGTTCAGGAG 300
EF614247        CAAAATACAGTACCTTTGGAAACGGTTAAAACGAATAAGAAAAATGTAACTGTTCAGGAG 297
                ************************************************************

EF614245        TTGGATCTTCAAGCAAGACGTTATTTACAGGAAAAATATAATTTATATAACTCTGATGTT 354
EF614246        TTGGATCTTCAAGCAAGACGTTATTTACAGGAAAAATATAATTTATATAACTCTGATGTT 360
EF614247        TTGGATCTTCAAGCAAGACGTTATTTACAGGAAAAATATAATTTATATAACTCTGATGTT 357
                ************************************************************

EF614245        TTTGATGGGAAGGTTCAGAGGGGATTAATCGTGTTTCATACTTCTACAGAACCTTCGGTT 414
EF614246        TTTGATGGGAAGGTTCAGAGGGGATTAATCGTGTTTCATACTTCTACAGAACCTTCGGTT 420
EF614247        TTTGATGGGAAGGTTCAGAGGGGATTAATCGTGTTTCATACTTCTACAGAACCTTCGGTT 417
                ************************************************************

EF614245        AATTACGATTTATTTGGTGCTCAAGGACAGAATTCAAATACACTATTAAGAATATATAGA 474
EF614246        AATTACGATTTATTTGGTGCTCAAGGACAGTATTCAAATACACTATTAAGAATATATAGA 480
EF614247        AATTACGATTTATTTGGTGCTCAAGGACAGTATTCAAATACACTATTAAGAATATATAGA 477
                ****************************** ****************************

EF614245        GATAATAAAACGATTAACTCTG  496
EF614246        GATAATAAAACGATTAACTCTG  502
EF614247        GATAATAAAACGATTAACTCTG  499
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Fig. 6: Nucleotide sequence alignment of *sea* gene for three *S. aureus* strains. Nucleotide changes are light blue color

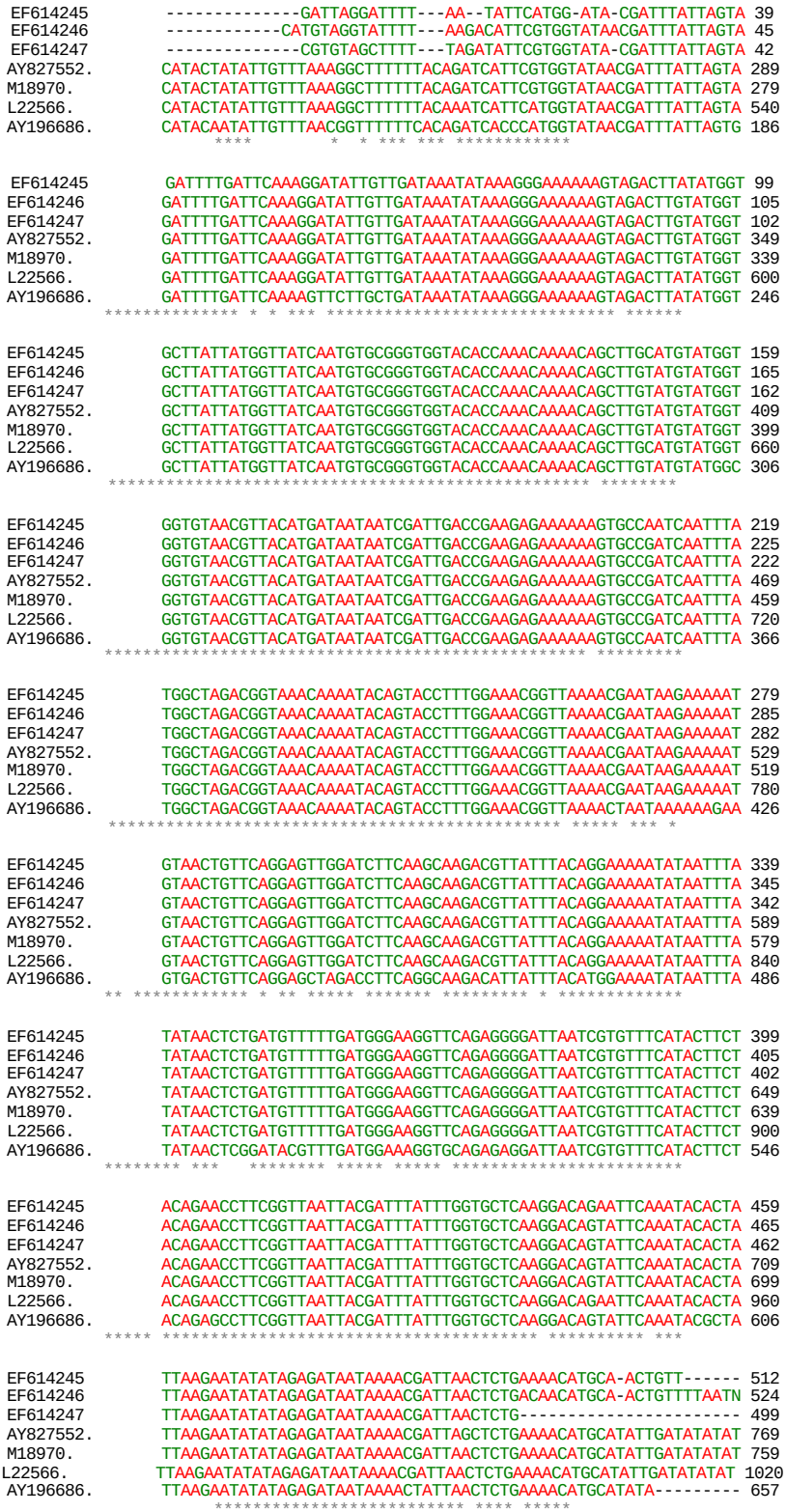

Fig. 7: The alignment of *sea* gene of *S. aureus* with the published sequence in GenBank stars indicates the similarity between isolates

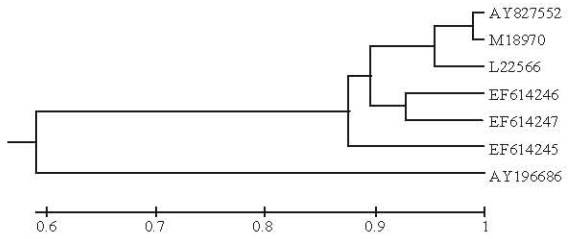

Fig. 8: Phylogenetic tree for comparative of these sequences with other sequences in GenBank {trees were constructed by the neighbour-joining (NJ) method using MEGA version 3.1 (Kumar *et al*. 2004)}

isolates when compared together (Fig. 6). BLAST search at the GenBank database with the *sea* sequences for other species of *S. aureus* displayed EF614246 and EF614247 clearly closely related to L22566, M18970 and AY827552 with nucleotide sequence identity ranged between (90-97%), all form one cluster indicating a high degree of homology between partial sequences of *sea* sequences of these strains. Whereas, the alignment score comparing EF614245 isolate with all other isolates ranged between 87-90% as shown in Fig. 7. Phylogenetic construction was carried out using MEGA 3.1 based on the DNA nucleotide sequence of the *sea* genes for all the examined isolates as shown in Fig. 8.

DISCUSSION

S. aureus produce a large number of substances that are involved in promoting the disease state. These virulence factors include both exoproteins, such as secreted toxins and factors that play diverse roles in pathogenesis and responsible for a variety of mild to life-threatening infections [5]. In the present study, 22% of the isolates harboured *sea* and 2% of the isolates harboured both *seb* and *sec* genes. The prevalence of classical sea-see genes was reported in other countries [7, 14]. The variation in prevalence of enterotoxin genes might be due to geographical differences, ecological origin of strains, the sensitivity of detection methods and number of samples included in the study [7, 12, 17]. In this study, *sea* gene was found to be dominating among strains. Similar results have been reported among strains from human source including strains causing food poisoning [7, 12].

The prevalence of the newly described SE genes (*seg-sej*) among isolates as follows: 16% *seg*, 12% *sei* and 1% for both *she* and *sej*. This result indicates that the *seg* and *sei* genes were dominant and often associated with each other in the tested clinical *S. aureus* isolates. Similar results have been reported in Germany [7], France [9], Korea [39] and Taiwan [40]. The combination of *seg* and *sei* was reported in 12% of Egyptian clinical isolates could be because these genes are components of the *egc* operon (*seg*, *sei*, *sem*, *sen* and *seo*) [8]. Similar results concerning *seg* and *sei* combination was reported in the Japanese (16.9%) [14]. In contrast, a higher coexistence rate was reported in the German multicenter (53%) [7]. In the present study, the *sej* gene was detected without *sed* and this suggests that another unknown mobile element may be involved with the *sed* gene. The prevalence of *sej* and *sed* was reported by many investigators [7, 13, 41]. Also, The presence of the classical SE genes *sea* or both *seb* and *sec* were detected simultaneously with the presence of the newly described SE genes *seg* and *sei* in 3 (6%) out of the 50 tested strains, while nine (18%) of isolates harbored classical SE gene(s) only and six (12%) of isolates harbored newly described SE gene(s) (Table 2 and Fig. 3).

The 50 clinical *S. aureus* isolates were typed by RAPD in order to determine the clonal relatedness of the isolates and to test whether SE positive strains are genetically clustered. Typing by RAPD assay using the summed result for three primers revealed that the 50 clinical *S. aureus* isolates were genetically diverse and comprised a heterogeneous population with 7 genotypes at a 65% similarity level. Genotype VII appeared to be predominant. Genotypes I and II included only *sea*-positive isolates (Table 2), while genotypes III, IV,V and VII included a great diversity of enterotoxigenic isolates (*sea*, *seb*, *sec*, *seg*, *she*, *sei* and *sej*). The presence of these genes was not genotype specific. In agreement with our results, Fueyo *et al.* [17] reported that some SE-positive and some SE-negative strains generated identical RAPD banding profiles, suggesting that SE-positive strains do not belong to a specific genetic class. In addition, Araki *et al.* [42] showed that there was no association between RAPD genotypes and the presence of toxin genes.

The genotypes obtained by PCR-RFLP of 16s rRNA gene was carried out and the amplified fragments (370 bp) were digested using five restriction endonucleases. Only HindIII was able to recognized different restriction sites along the examined genes and produced three different types A, B and C for all tested strains. Genotype A appeared to be predominant. 40% of the *S. aureus* isolates

belonged to type A, 32% to type B and 28% to type C. Most and great diversity of enterotoxigenic isolates belonged to genotype B (Fig. 5). Whenever, PCR-RFLP using TaqI RsaI, AluI and MspI enzymes showed identical restriction patterns with all tested isolates. These results were in accordance with Nema *et al.* [26] (Fig. 4). Although the identified *S. aureus* genotypes harbored the tested SE genes either singly or in combination, X^2 test with ($P<0.05$) showed that the overall presence of the enterotoxin genes is independent of the genotypes obtained by PCR-RFLP of 16s rRNA gene.

The combined use of RAPD and 16s rRNA PCR-RFLP techniques demonstrates that most (18) isolates in predominant RFLP genotype A clustered separately within the predominant RAPD genotype VII. On the other hand, isolates of genotype A were clustered within 3 RAPD genotypes (I, II, III and IV). In addition, isolates of genotype C clustered within 2 RAPD genotypes (V and VI). These results have also been supported by *sea* sequencing data of isolates *S. aureus* number 20 (type A), *S. aureus* number 9 (type B) and *S. aureus* number 29 (type A) that showed difference among themselves. More than 10 nucleotide variations were observed in the *sea* gene sequence of the isolates when compared with some as shown in (Fig. 6). This variations lead to change in translated amino acids. However, there were several variations in *sea* gene of isolates (EF614245; EF614246; EF614247) when compared with the known sequences (L22566; M18970; AY827552; AY196686) available in the GenBank (Fig. 7). Comparative analysis of these sequences showed that EF614246 and EF614247 clearly closely related to L22566, M18970 and AY827552 with nucleotide sequence identity ranged between (90-97%). Whereas, the alignment score comparing EF614245 isolate with all other isolates ranged between 87-90%. The high degree of sequence homology observed in the *sea* gene sequences of each species indicates that the high level of intraspecific homogeneity for each species.

In conclusion, the present investigation demonstrated that the *sea* gene was the predominant enterotoxin gene in these genetically diverse Egyptian clinical isolates. Further more, this work indicates a systematic association between *seg* and *sei* and a wide distribution of these two genes among the *S. aureus* strains. RAPD technique exhibits greater discriminatory power associated with 16s rRNA PCR-RFLP and describing their clonal relationships. Thus, DNA sequencing analysis is simple and useful methods to investigate the existence of regions for enterotoxin gene rearrangement in *S. aureus* and the phylogenetic aspects of the staphylococcal enterotoxins. In addition it is a good tool to determine the evolution of the studied organism as well as studying one or more genes inside the same organism.

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