Turkish Journal of Medical Sciences

Volume 41 | Number 2 Article 21

1-1-2011

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Original Article

Turk J Med Sci
2011; 41 (2): 343-352
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E-mail: medsci@tubitak.gov.tr
doi:10.3906/sag-1003-657

Investigation of toxin genes in *Staphylococcus aureus* strains isolated in Mustafa Kemal University Hospital

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Aim: The aim of this study was to investigate the presence of genes encoding staphylococcal enterotoxins (SEs), exfoliative toxins (ETAs, ETBs), and toxic shock syndrome toxin-1 (TSST-1) by polymerase chain reaction (PCR) in *Staphylococcus aureus* strains isolated from various clinical samples from the Mustafa Kemal University Hospital. In addition, PCR-based restriction fragment length polymorphism (RFLP) analysis of the coa gene was employed to genotype the isolates.

Materials and methods: A total of 120 *S. aureus* strains isolated from various clinical samples (blood, wounds, urine, conjuctival swabs, and tracheal aspirate) over a 1 year period, 2007-2008, were used in this study.

Results: Almost 65.8% of the isolates possessed at least one toxin gene. The genes most frequently found were *seg-sei* (40.8%), followed by *sea* (30%) and *eta* (19.2%). Overall, 35 toxin genotypes were observed, among which the genotypes *seg-sei, sea-seg-sei,* and *sea-see* predominated at the rate of 8.3%, 5.8%, and 5%, respectively. Four coagulase genotype patterns were observed, with molecular sizes ranging from 570 to 970 bp. *Coa*-based RFLP analysis revealed 7 different patterns using *Alu*I.

Conclusion: Our results have revealed that toxin genes were very prevalent among *S. aureus* isolates, and the toxigenic isolates were independent of the genotypes obtained by PCR-RFLP of the coa gene (P > 0.05).

Key words: *Staphylococcus aureus*, toxin genes, PCR-RFLP, coagulase

Mustafa Kemal Üniversitesi Hastanesi'nde izole edilen *Staphylococcus aureus* suşlarında toksin genlerinin araştırılması

Amaç: Bu çalışmanın amacı Mustafa Kemal Üniversitesi Hastanesi'nde farklı klinik örneklerden izole edilen *Staphylococcus aureus* suşlarında stafilokokkal enterotoksinler (SE), eksfoliyatif toksinler (ETA, ETB) ve toksik şok sendrom toksin-1'i (TSST-1) kodlayan genlerin Polimeraz Zincir Reaksiyonu (PZR) ile belirlenmesi ve koagulaz genine dayalı restriction fragment length polymorphism (PZR-RFLP) ile tiplendirilmesidir.

Yöntem ve gereç: Çalışmada farklı klinik örneklerden (kan, yara, idrar, konjuktival sürüntü örneği ve trakeal aspirat) 2007-2008 yılları arasında izole edilen 120 *S. aureus* suşu kullanıldı.

Bulgular: İzolatların % 65,8'inin en az bir toksin geni taşıdığı saptandı. En yaygın toksin genlerinin *seg-sei* (% 40,8), *sea* (% 30) ve *eta* (% 19,2) olduğu tespit edildi. Çalışmada 35 toksin genotipinden, *seg-sei* (% 8.3), *sea-seg-sei* (% 5,8) ve *sea-see* (% 5) en yaygın genotipler olarak belirlendi. Moleküler büyüklükleri 570-970 bp arasında değişen 4 koagulaz genotipi gözlendi. Koagulaz geninin *Alu*I ile kesilmesi ile yapılan RFLP analizi ile 7 farklı pattern bulundu.

Sonuç: *S. aureus* izolatları arasında toksin genlerinin oldukça yaygın olduğu ve toksijenik suşların *coa* genine dayalı yapılan PZR-RFLP ile elde edilen genotiplerden bağımsız olduğu sonucuna varılmıştır (P > 0,05).

Anahtar sözcükler: *Staphylococcus aureus*, toksin genleri, PZR-RFLP, koagulaz

Received: 03.03.2010 – Accepted: 19.07.2010

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Introduction

Clinical isolates of *Staphylococcus aureus* can produce a variety of extracellular toxins, which are thought to contribute to the pathogenicity of the organism. Staphylococcal enterotoxins (SEs), toxic shock syndrome toxin-1 (TSST-1), and exfoliative toxins (ETs) produced by some *S. aureus* isolates causes staphylococcal food poisoning (SFP), staphylococcal toxic shock syndrome (TSS), and staphylococcal scalded-skin syndrome (SSSS) (1). Both SEs and TSST-1 are included in the pyrogenic toxin superantigen families (PTSAgs). PTSAgs exert their virulence by binding to the major histocompatibility complex (MHC) class II molecules and the Vβ chain of the T-cell receptor (TCR) from the outside in a nonspecific manner. This leads to the stimulation of T-cell proliferation, the release of inflammatory cytokines, and ultimately the suppression of the host immune system (2). According to the International Committee for Staphylococcal Superantigens Nomenclature (INCSSN), only staphylococcal SAgs that induce emesis after oral administration in a primate model should be designated as SEs. Other related toxins that either lack the emetic properties in this model or have not been tested should be designated as staphylococcal enterotoxin-like (SEI) superantigens (3). In addition to 5 well-characterized classical staphylococcal enterotoxins SEA-SEE, 14 new types of SEs (SEG, SEH, and SEI) or SEIs (SEIJ, SEIK, SEIL, SEIM, SEIN, SEIO, SEIP, SEIQ, SEIR, and SEIU) have been reported (4-12).

Many molecular methods have been described for typing *S. aureus* isolates (13). Among these, polymerase chain reaction (PCR) and PCR-based restriction fragment length polymorphism (PCR-RFLP) of the 3' end of the gene encoding the staphylococcal coagulase (*coa*) gene have been proposed as methods for typing *S. aureus* isolates for epidemiological studies. Amplified DNA fragments of different sizes can be further discriminated through digestion with *Alu*I(14).

Epidemiological studies for the investigation of classical and newly-described PTSAg and ET genes are lacking in Turkey. In this study we investigated the prevalence of PTSAgs and ETs in *S. aureus* strains isolated from different clinical samples and the genetic variation among isolates using PCR-RFLP analysis based on the *coa* gene.

Materials and methods

Bacterial isolates

One hundred and twenty clinical *S. aureus* isolates were randomly collected from wounds (n: 48), urine (n: 36), blood (n: 17), tracheal aspirate (n: 11), and conjuctival swabs (n: 8) over a 1-year period from 2007-2008. Specimens were submitted to the Microbiology Laboratory at the Mustafa Kemal University Hospital, Faculty of Medicine.

DNA isolation

Bacterial DNA was isolated as described by Hesselbarth and Schwarz (15) with some modifications. Briefly, *S. aureus* isolates were grown in 10 mL of brain heart infusion (BHI) broth at 37 °C overnight under aerobic conditions. Bacterial cells were collected through centrifugation for 10 min at 3500 rpm, washed in 1 mL of TES buffer (10 mM Tris·HCl, pH 8.0; 1 mM EDTA; 100 mM NaCl), and re-centrifuged. The pellet was re-suspended in 500 µL of TES buffer including 2 µL of lysostaphin (2 µg/mL, Sigma-Aldrich, St. Louis, MO, USA) and 10 µL of lysozyme (1 mg/mL, AppliChem, Darmstadt, Germany) and incubated for 30 min at 37 °C. Then, for complete lysis, 20 µL of 10% SDS solution was added and incubated for 15 min at room temperature. The bacterial DNA was extracted with equal volumes of phenol-chloroform-isoamyl alchohol (25:24:1) and chloroform-isoamyl-alchohol (24:1). The DNA was precipitated from the aqueous phase with an equal volume of isopropanol at –20 °C overnight. After the final centrifugation, the supernatant was discarded. The pellet was washed with 70% ethanol, dried, and re-suspended in 100 µL of DEPC-treated water.

Detection of toxin genes by PCR

The nucleotide sequences of all primers used in this study and their respective amplified products are listed in Table 1. As reported by Mehrotra et al. (16) and Monday and Bohach (17), 3 sets of multiplex PCR were performed for the detection of toxin genes. Set A contained 200 µM dNTP mix, 5 µL of 10 × PCR buffer [100 mM Tris-HCl (pH 8.3), 500 mM KCl], 1.5 mM $MgCl_2$, 20 pmol each of *sea*, *seb*, *sec*, *see*, and *femA* primers, which is essential for methicillin resistance and is universally present in all *S. aureus* isolates, 40 pmol of *sed* primer, 2.5 U of *Taq* polymerase (Fermentas), and 5 µL of template DNA.

Table 1. Oligonucleotide primers used for the amplification of the toxin genes and *coa* gene.

Gene	Oligonucleotide sequence (5'-3')	Product size (bp)	Reference
sea	GGTTATCAATGTGCGGGTGG	102	Mehrotra et al. (16)
	CGGCACTTTTTTCTCTTCGG		
seb	GTATGGTGGTGTAACTGAGC	164	Mehrotra et al. (16)
	CCAAATAGTGACGAGTTAGG		
sec	AGATGAAGTAGTTGATGTGTATGG	451	Mehrotra et al. (16)
	CACACTTTTAGAATCAACCG		
sed	CCAATAATAGGAGAAAATAAAAG	278	Mehrotra et al. (16)
	ATTGGTATTTTTTTTTCGTTC		
see	AGGTTTTTTCACAGGTCATCC	209	Mehrotra et al. (16)
	CTTTTTTTTCTTCGGTCAATC		
femA	AAAAAAGCACATAACAAGCG	132	Mehrotra et al. (16)
	GATAAAGAAGAAACCAGCAG		
eta	GCAGGTGTTGATTTAGCATT	93	Mehrotra et al. (16)
	AGATGTCCCTATTTTTGCTG		
etb	ACAAGCAAAAGAATACAGCG	226	Mehrotra et al. (16)
	GTTTTTGGCTGCTTCTCTTG		
tst	ACCCCTGTTCCCTTATCATC	326	Mehrotra et al. (16)
	TTTTCAGTATTTGTAACGCC		
seg	CGTCTCCACCTGTTGAAG G	327	Monday et al. (17)
	CCAAGTGATTGTCTATTGTCG		
seh	CAACTGCTGATTTAGCTCAG	360	Monday et al. (17)
	GTCGAATGAGTAATCTCTAGG		
sei	CAACTCGAATTTTCAACAGGTAC	465	Monday et al. (17)
	CAGGCAGTCCATCTCCTG		
selj	CATCAGAACTGTTGTTCCGCTAG	142	Monday et al. (17)
	CTGAATTTTACCATCAAAGGTAC		
coa	CGAGACCAAGATTCAACAAG	Variable	Goh et al. (14)
	AAAGAAAAACCACTCACATCA		

The volume of this mix was adjusted to 50 µL with sterile water. Set B contained the same constituents as set A with the exception of the $MgCl_2$ concentration (2.0 mM) and primers, which were used at 50 pmol for *eta* and 20 pmol for *etb* and *tst*. Set C was performed in a 50 µL volume containing the following: 5 µL of 10 × PCR buffer [100 mM Tris-HCl (pH 8.3), 500 mM KCl], 4 mM $MgCl_2$, 50 pmol concentrations each of *seg*, *seh*, *sei*, and *selj* primers, 400 µM dNTP, 5 U of *Taq* polymerase, and 5 µL of template DNA. PCRs were carried out in a Techne TC-312 thermal cycler (Techne, USA). Amplifications of set A and set B mixtures were carried out following cycling conditions: an initial denaturation at 94 °C for 5 min was followed by 35 cycles of denaturation at 94 °C for 2 min, annealing at 57 °C for 2 min, an extension at 72 °C for 1 min, and ending with a final extension at 72 °C for 7 min. Set C mixture was amplified by 15 cycles of 95 °C for 1 min, 68 °C for 45 s, and 72 °C for 1 min and 16 cycles of 95 °C for 1 min, 64 °C for 45 s, 72 °C for 1 min, and then one cycle was performed at 72 °C for 10 min of extra extension. Amplified products were resolved by electrophoresis at 100 V for 1 h in 1.5% agarose gel, stained with ethidium bromide (0.5 µg/mL), and visualized by UV-transilluminator.

Genotyping of *S. aureus* by PCR-RFLP analysis of the *coa* gene

Amplification of the *coa* gene of *S. aureus* by PCR-RFLP was performed as described by Goh et al. (14). Briefly, PCR was performed in a 50 µL volume containing 5 µL of 10 × PCR buffer [750 mM Tris HCl, pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], 200 µM of dNTP mixture, 1.25 U of *Taq* polymerase, 50 pmol of each primer, and 5 µL of template DNA. Amplification of DNA was performed using the following cycling conditions: pre-denaturation at 95 °C for 2 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 2 min, extension at 72 °C for 2 min for 30 cycles. A 10 min extension step at 72 °C was included at the end of the final cycle. Amplified products were resolved in a 1.5% agarose gel in 1 × TBE buffer at 100 V and visualized with ethidium bromide. After electrophoresis, 10 µL of product was incubated overnight with 2 U of *Alu*I restriction endonuclease and 1.5 µL of 10 × restriction buffer according to the manufacturer's instructions. The resulting fragments were separated on 2% agarose gel 110 V for 1 h, stained with ethidium bromide (0.5 µg/mL), and visualized under UV light.

Statistical analysis

The relationship between toxin genes and the genotypes of *S. aureus* isolates were assessed using Statistical Package for the Social Sciences (SPSS) 11.0. Values of $P < 0.05$ were considered statistically significant.

Results

Detection of toxin genes

The prevalence of toxin genes found in 120 clinical *S. aureus* isolates collected from various clinical samples by multiplex PCR are shown in Table 2. Of 120 isolates investigated, 79 (65.8%) were found to be positive for one or more toxin genes. Among the *S. aureus* isolates obtained from wound (n: 48), urine (n: 36), blood (n: 17), tracheal aspirate (n: 11), and conjuctival swab (n: 8) specimens, 40 (83.3%), 18 (50%), 12 (70.6%), 6 (54.5%), and 3 (37.5%) were positive for at least one toxin gene, respectively. The most commonly found genes were the recently-described enterotoxin genes *seg* and *sei* (n:49, 40.8%), which were found together with other toxin genes. However, 11 isolates harbored these genes alone. Among the classical enterotoxin genes *sea* gene (n: 36, 30%) was the most widespread, followed by *sec* (n: 9, 7.5%), *sed* (n: 6, 5%), and *see* (n: 10, 8.3%). Positive isolates of the *sed* gene are always found in combination with the newly described enterotoxin gene *selj*. Six isolates (5%) were diagnosed as carriers of the *sed* and *selj* genes together.

Multiple toxin gene combinations were observed including 2 (n: 25, 20.8%), 3 (n: 18, 15%), 4 (n: 11, 9.2%), 5 (n: 9, 7.5%), and 6 (n: 3, 2.5%) different genes. The most frequent combination detected was *seg-sei* in 11 (13.9%) isolates, *sea-seg-sei* in 7 (8.9%) isolates, and *sea-see* in 6 (7.6%) isolates.

Among the 120 isolates examined 34 (28.3%) isolates were positive for 1 or 2 ET genes tested. Of these, *eta* was found alone in 5 isolates, and together with *etb* in 1 isolate. However, ET gene combinations mostly carried the *seg-sei* gene combination.

Table 2. Distribution of toxin genes in *S. aureus* from different human clinical specimens.

Gene combination	**No. of *S. aureus* isolates**					**Total**
	Wound (n: 48)	**Urine (n: 36)**	**Blood (n: 17)**	**Tracheal aspirate (n: 11)**	**Conjuctival swabs (n: 8)**	
sea	3		1		1	5
seb			1			1
tst		2				2
eta	3	2				5
sea, eta	1			1		2
sea, see	3	1	2			6
sea, seb	1					1
sea, tst					1	1
sea, seh				1		1
tst, eta		1				1
eta, etb		1				1
seb, seh		1				1
seg, sei	5	3	2		1	11
sea, seg, sei	3	2	1	1		7
sea, seb, seh		1				1
sec, seg, sei		1	1			2
seb, seg, sei				1		1
seg, sei, eta	2					2
seg, sei, tst	1	1	1	1		4
tst, eta, etb			1			1
sed, seg, sei, selj	1					1
seg, sei, eta, etb	2	1		1		4
sea, sec, seg, sei	1					1
sea, sed, see, selj	1					1
sea, seg, sei, tst	1					1
sed, see, seh, selj	1					1
sec, seg, sei, tst	1	1				2
sed, seg, sei, selj, eta	1					1
sea, see, seg, sei, tst	1					1
sea, sec, seg, sei, tst	2					2
sea, seg, sei, eta, etb	2		1			3
sea, sec, seg, sei,eta	1		1			2
sea, see, seg, sei, seh, tst	1					1
sed, seg, sei, selj, eta, etb	1					1
sed, seg, sei, selj, tst, etb	1					1
Total negative	**8**	**18**	**5**	**5**	**5**	**41**
Total positive	**40**	**18**	**12**	**6**	**3**	**79**

PCR-RFLP analysis of *coa* gene

All isolates produced a single band in *coa* PCR, with molecular sizes ranging from 570 to 970 bp (Figure 1). A PCR product with an approximate size of 970 bp was the most common profile and was produced by 32 (26.7%) of the isolates. *Alu*I digestion of *coa*-positive PCR products yielded 7 different profiles. The number of fragments generated by *Alu*I varied from 1 to 3, and their sizes were between 80 and 490 bp (Figure 2). Among the isolates the main RFLP genotypes were I (26.7%), II (23.3%), III (3.3%), IV (14.2%), V (23.3%), VI (8.3%), and VII (0.8%). When RFLP genotypes and toxin genes of isolates were compared, no difference was observed (Table 3).

Relationship between the presence of toxin genes and the genotypes obtained by PCR-RFLP

The presence of toxin genes was independent ($P > 0.05$) of the genotypes of the *S. aureus* isolates tested.

Discussion

Multiplex PCR results obtained in this study showed that 79 isolates (65.8%) were positive for one or more toxin genes. Among enterotoxin genes, 69 isolates (57.5%) harbored one or more enterotoxin genes. This prevalence rate is higher than reported in German clinical (43%) and nasal (39.5%) isolates (18), in clinical isolates (23%) in Jordan (19), and in isolates (55.8%) from Taiwanese patients with food poisoning (20), but lower than that in isolates collected from Japanese (76%) patients with food poisoning (8). Recently, Tekeli et al. (21) reported a higher prevalence of SE genes (86%) in methicillin-resistant *S. aureus* (MRSA) strains isolated from the blood cultures of patients in Ankara, Turkey. This could be due to geographical differences, which may be further affected by the different ecological origins of the isolated strains (food, humans, and animals) (16,18,22).

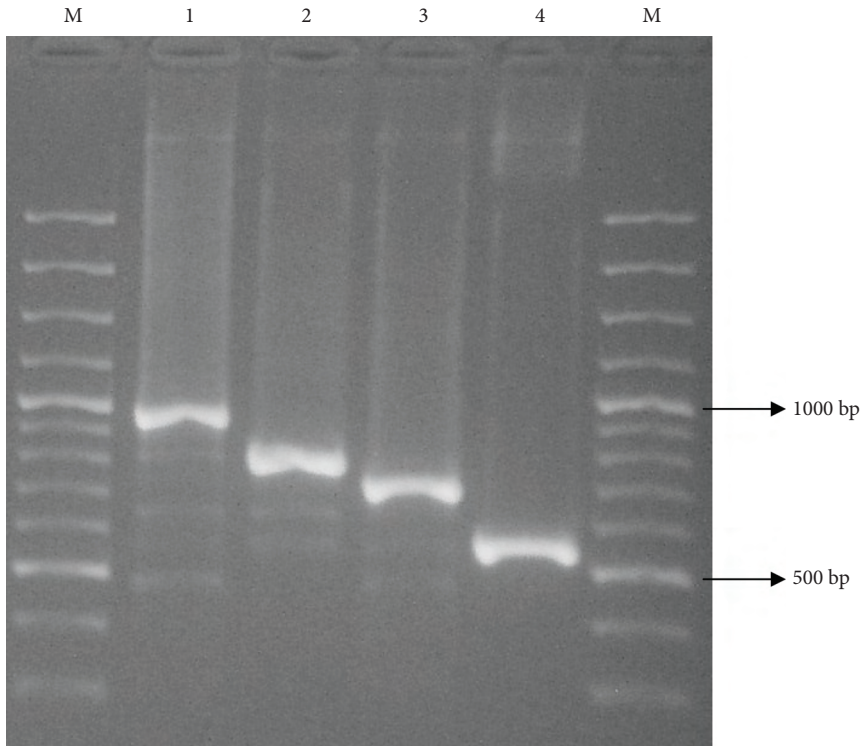

Figure 1. Agarose gel electrophoresis of *coa* PCR products of *S. aureus* isolates. M: 100 bp molecular weight marker, lane 1: amplicon of 970 bp, lane 2: amplicon of 810 bp, lane 3: amplicon of 730 bp, lane 4: amplicon of 550 bp.

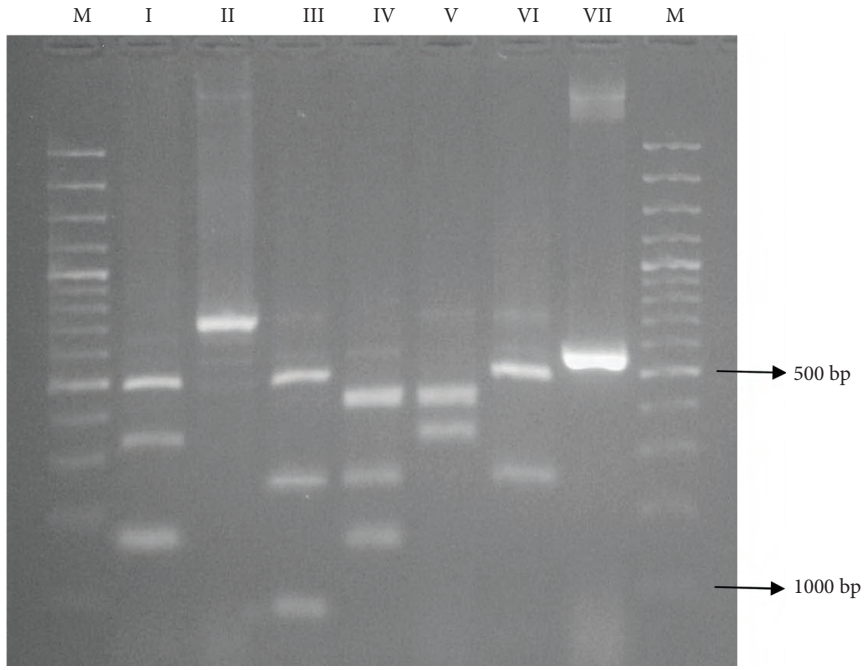

Figure 2. *Alu*I digested *coa* PCR products. M: 100 bp molecular weight marker. I-VII: Different RFLP patterns of the *coa* gene.

The predominant enterotoxin types in this study were *seg* and *sei*. These 48 isolates (40%) harbored the *seg* and *sei* combination in 11 isolates (9.2%) and in further combination with other toxin genes (30.8%). Similarly, other studies (18,23,24) showed that *seg* and *sei* genes are the dominant clinical isolates. The coexistence of *seg* and *sei* in *S. aureus* isolates could be explained by the localization of the enterotoxin gene cluster, *egc*, including *seg-sei-selm-seln-selo* and sometimes *selu*, which is located on the genomic island Type II v Saβ (11). These results regarding the *seg* and *sei* combination are in agreement with those of other investigators (8,11,18).

Similar coexisting gene combination was also reported for the *sed* and *selj* genes, because the SED encoding plasmid, pIB485, carries and encodes the *selj* gene (25). In the present study the *sed-selj* gene combination was detected in 6 isolates (5%). The same observation was reported by several investigators (18,20,26).

Seventeen isolates (14.2%) harbored *tst* in various combinations, including *sea-seg-sei*, *seh-seg-sei* and several other gene groupings. In contrast, 2 (1.7%) isolates were observed with *tst* only. This distribution rate (14.2%) was lower than the rate found in German blood (18.3%) and nasal (22.4%) *S. aureus* samples (18). Interestingly, a prevalence rate as high as 59.1% was reported in *S. aureus* isolates collected from staphylococcal food-poisoning outbreaks in Taiwan (20).

In this study, *eta* and *etb* genes were observed in 19.2% (n: 23) and 9.2% (n: 11) of the isolates examined, respectively. Although there are a few studies that detect ET genes in human-origin *S. aureus* isolates, our detection rates for ET genes were noticeably higher than those of other investigators (18,26,27).

Staphylococcal SAg genes and TSST-1 genes are encoded by mobile genetic elements (MGEs), such as pathogenicity islands (SaPIs), prophages, transposons, and plasmids (26,28). This fact implies that SAg genes are transferred horizontally between staphylococcal isolates. MGEs could account for the high isolation rate and wide distribution of SAg genes among isolates tested in this study.

Table 3. Distribution of toxin genes and PCR-RFLP genotypes of *S. aureus* isolates obtained from human clinical specimens.

Toxin type	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**
sea	3	0	0	2	0	0	0
seb	1	0	0	0	0	0	0
tst	1	0	1	0	0	0	0
eta	0	0	0	3	2	0	0
sea, eta	2	0	0	0	0	0	0
sea,see	0	6	0	0	0	0	0
sea, seb	0	1	0	0	0	0	0
sea, tst	0	0	0	0	1	0	0
sea, seh	0	0	0	0	1	0	0
tst, eta	0	0	0	0	0	1	0
eta, etb	0	1	0	0	0	0	0
seb, seh	1	0	0	0	0	0	0
seg, sei	2	4	0	0	2	2	1
sea, seg, sei	0	1	1	2	2	1	0
sea, seb, seh	0	1	0	0	0	0	0
sec, seg, sei	0	0	0	0	2	0	0
seb,seg, sei	0	0	0	0	1	0	0
seg, sei, eta	0	0	0	0	2	0	0
seg, sei, tst	0	1	1	1	1	0	0
tst, eta, etb	0	0	0	1	0	0	1
sed, seg, sei, selj	0	0	0	0	0	1	0
seg, sei, eta, etb	1	2	0	0	0	1	0
sea, sec, seg, sei	1	0	0	0	0	0	0
sea, sed, see, selj	0	0	1	0	0	0	0
sea, seg, sei, tst	0	0	0	1	0	0	0
sed, see, seh, selj	0	0	0	0	1	0	0
sec, seg, sei, tst	0	0	0	0	2	0	0
sed, seg, sei, selj, eta	0	0	0	0	1	0	0
sea, see, seg, sei, tst	0	0	0	0	1	0	0
sea, sec, seg, sei, tst	0	0	0	0	2	0	0
sea, seg, sei,eta, etb	2	1	0	0	0	0	0
sea, sec, seg, sei, eta	2	0	0	0	0	0	0
sea, see, seg, sei, seh, tst	0	0	0	0	0	1	0
sed, seg, sei, selj, eta, etb	0	1	0	0	0	0	0
sed, seg, sei, selj, tst, etb	1	0	0	0	0	0	0
Total	**17**	**19**	**4**	**10**	**21**	**7**	**1**

Superantigen toxin and ET genes alone and/or in different combinations were detected in clinical *S. aureus* isolates by the PCR method. A high frequency rate, however, does not mean that these isolates produce toxin at a level sufficient to cause disease. For example, it was shown that *S. aureus* isolates from nasal swab samples harbored toxin genes at a high ratio, 47.4% (4) and 71.4% (18). Consequently, additional studies are needed to demonstrate the toxic levels of these isolates.

Our results demonstrated that SAg and ET genes were very prevalent among clinical *S. aureus* isolates, and the prevalence of these genes was not genotype specific. Because most of the SAg genes were encoded by certain MGEs, further studies should be carried out to detect the prevalence of new SAg genes and the characterization of possible new MGEs.

Acknowledgments

This study was supported by Mustafa Kemal University Scientific Research Projects (BAP- 05 G 0201).

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