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Sap11/Sap12 and *egc* Associated Toxin Genes are Dominant in Slime Forming Clinical Staphylococcus Aureus Isolates Harboring *icaABCD-Operon*

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Abstract

The prevalence of adhesion (*icaA* and *icaD*) and toxin (*tst, eta,* and *etb*) genes was studied in 100 clinical *Staphylococcus aureus* isolates. The *icaA, icaD*, *eta*, *etb* and *tst* genes were detected by PCR in 91%, 91%, 2%, 1% and 43% of the isolates, respectively. Various gene combinations with the previously reported enterotoxin genes (*sea*, *sec*, *sei*, *seg* and *seh*) were detected in 47 of 100 isolates. These combinations fall into 3 groups: group I which includes one *eta*+*sea* containing isolate, group II which includes 25 (53.2%) isolates lacking *tst* gene but harboring *icaA*+*icaD* genes in 11 combinations with the enterotoxin genes and group III which includes 21 (44.7%) isolates harboring *icaA*+*icaD*+*tst* in 7 different combinations with the enterotoxin genes. The predominant gene combinations in group II and III isolates include *seg* + *sei* or *seg* of the enterotoxin gene cluster (*egc*). Furthermore, 20 isolates of group III have the corresponding gene combination profiles of 20 isolates of group II in addition to the *tst* gene. This could be attributed to loss or acquisition of SaPI1 and/or SaPI2 islands which carry the *tst* gene.
The dominancy of SaPI1/SaPI2 and *egc* associated toxin genes in the slimy isolates of *S. aureus* may be understood in the context of pathogenicity functioning genes. This data would contribute to the control of colonization and spread of these isolates in hospitals and community at large.

الملخص

في هذه الدراسة تحديد جينات الالتصاق من فئة icaA, icaD ، وكذلك جينات السمية من فئات efb و eta و tst في 100 عزلة سريرية من البكتيريا العنقودية الذهبية S.aureus وقد تم وجود جينات icaA و icaA و etb و tst بنسب 91، 91، 2، 1، 43% في العزلات على التوالي وذلك باستعمال تقنية تفاعل انزيم البوليمريز المتسلسل (PCR) . كما ولوحظ وجود توليفات من جينات مختلفة مع تلك الموجودة في الأدب العلمي من فئات seg, sei, sec, sea seh في 47 عزلة من المئة التي تمت دراستها. وتقع هذه التوليفات في 3 مجموعات. المجموعة الأولى والتي تشمل عزلة واحدة تحوي الجينات sea + eta والمجموعة الثانية تشمل 25 عزلة تفتقر للجين tst إلا أنها تحمل الجينات icaA + icaD متمثله في 11 توليفة من جينات السمية أما المجموعة الثالثة فتشمل 21 عزلة تحمل جينات icaA + icaD + tst موزعة في سبع توليفات مع جينات السمية. أما التوليفات السائدة في المجموعتين الثانية والثالثة فتشمل sei + seg أو seg من المجموعة الجينية السمية egc. إضافة لذلك فإن عشرين عزلة من المجموعة الثالثة تحمل أنماط التوليفات المعادلة في عشرين عزلة من المجموعة الثانية إضافة للجين tst. ويمكن أن نغزي ذلك إما الى حمل أو فقد الجزر المرضية من SaPI1 و أو SaPI2 التي تحمل جين tst.
إن سيادة جينات SaPI2 و SaPI2 وجينات السمية المرتبطة بجين ege في عزلات المكورات العنقودية الذهبية الفروية يمكن فهمه في سياق الجينات الامراضية الفاعلة ويمكننا القول بأن نتائج هذه الدراسة سوف تساهم في السيطرة على التموضع وانتشار مثل هذه العزلات في بيئات المستشفيات والمجتمع بصورة عامة.

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1. Introduction

Staphylococcus aureus is an important pathogen causing a variety of diseases in both human and animals (Archer, 1998; Vasudevan *et al*., 2003). Its pathogenesis is attributed to combined effect of toxins and extracellular factors encoded by different genes. These include: superantigens [enterotoxins (SEs), toxic shock syndrome toxin-1 (TSST-1), exfoliative toxins (ETA and ETB)], fibronectin, collagen and fibrinogen binding proteins, in addition to proteins involved in biofilm formation (Baron *et al*., 1994; Palma *et al*., 1996, 1998; Becker *et al*., 1998; Cramton *et al*., 1999; Jarraud *et al*., 2001; Peacock *et al*., 2002; Cucarella *et al*., 2004; Fueyo *et al*., 2005).

Biofilm formation requires production of the extracellular poly-*N*-acetylglucosamine (PNAG) by *icaABCD*-operon encoded enzymes (Cramton *et al*., 1999; Fitzpatrick *et al*., 2005a,b). The *icaA* gene encodes *N*-acetylglucosaminyltransferase which is involved in the synthesis of *N*-acetylglucosamine oligomers from UDP-*N*-acetylglucosamine (Arciola *et al*., 2001a).

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The *icaD* gene is involved in expression of *N*-acetylglucosaminyltransferase (Gerke *et al*., 1998). *S. aureus* isolates harboring the *ica* gene cluster are responsible for chronic or persistent infections which can be more problematic due to the presence of antibiotic resistance genes (Stewart and Costerton, 2001; Victoria *et al*., 2002).

Since virulent *S. aureus* strains pose a real problem by increasing healthcare cost worldwide, both in hospitals and in community (Costerton *et al*., 1999), these strains have been identified in several countries in the quest of controlling their spread (Omoe *et al*., 2002; Peacock *et al*., 2002; Becker *et al*., 2003). In previous studies, the prevalence of the staphylococcal enterotoxin genes (*sea*-*see* and *seg*-*sej*) was investigated in Jordanian clinical *S. aureus* isolates. Only *sea*, *sec*, *seg*, *sei* and *seh* were detected in 15%, 4%, 37%, 24% and 4% of the total isolates, respectively (Naffa *et al*., 2006; El-Huneidi *et al*., 2006). In this study, we followed the presence of toxic shock syndrome (*tst*), exfoliative toxin (*eta* and *etb*), and the adhesion (*icaA* and *icaD*) genes in the above mentioned isolates. Targeting such additional genes provides further epidemiological information on the toxigenicity of the Jordanian isolates and their ability to form biofilms. This information may also contribute to the control of colonization and the spread of these isolates in the Jordanian hospitals and community.

2. Materials and Methods

2.1. Bacterial Isolates.

A total of 100 clinical *S. aureus* isolates recovered from patients with invasive *S. aureus* diseases admitted to the Jordan University Hospital (Al-Zu'bi, 2004), were included in this study. All isolates were identified by biochemical tests (Daghistani *et al*., 2000).

The following *S. aureus* reference strains were used as positive controls for the studied genes: CECT 975 (*icaA* and *icaD* positive) was kindly provided by the Spanish Type Culture Collection (CECT); *S. aureus* E-1 (*eta* positive) and TY4 (*etb* positive) strains were kindly provided by Dr. Motoyuki Sugai, Graduate School of Biomedical Sciences, Dept. of Bacteriology, Hiroshima University, Hiroshima-Japan; NCTC 11963 (*tst* positive) was purchased from the National Collection of Type Culture (NCTC).

2.2. Slime-Production

The phenotypic ability of *S. aureus* isolates for slime-production was determined by cultivation on Congo red agar (CRA) plates (Freeman *et al*., 1989; Montanaro *et al*., 1999; Arciola *et al*., 2001a; Vasudevan *et al*., 2003; Cucarella *et al*., 2004). CRA plates were incubated for 24 h at 37°C and subsequently overnight at room temperature. Slime producing isolates form black colonies, whereas non-producing isolates develop red colonies. The result was confirmed by amplification of *icaA* and *icaD* genes using the polymerase chain reaction technique (PCR).

2.3. Detection of Adhesion and Toxin Genes

Cell lysate of *S. aureus* (Van de Klundert and Vligenthard, 1993) containing both chromosomal and plasmid DNA was used in 25 µl of PCR reaction mixture. Table 1 shows the sequence and the quantity of primers, amplification conditions and anticipated sizes of PCR products for the tested genes. PCR amplifications were performed in a PE-9600 thermocycler (Perkin-Elmer) using PCR Master Mix (Promega, USA). A positive PCR control containing cell lysate of a reference *S. aureus* and a negative PCR blank with nuclease free water instead of the cell lysate were included with each set of five reactions. After amplification, 10µl of each PCR mixture was analyzed in 1.5% agarose gel, and photographed using the Gel documentation system (UVP, USA).

2.4. Statistical Analysis

The correlation coefficient (r) between the slime producing isolates and the *icaA* and *icaD* gene harboring isolates was calculated using the correlation coefficient (r) formula. Test statistic was used to accept or reject the null hypothesis that there is a significant difference in prevalence of corresponding gene combinations in isolates. *P*-value was calculated at 95% confidence interval using normal distribution tables. $p < 0.05$ was considered statistically significant (Johnson and Bhattacharyya, 1996).

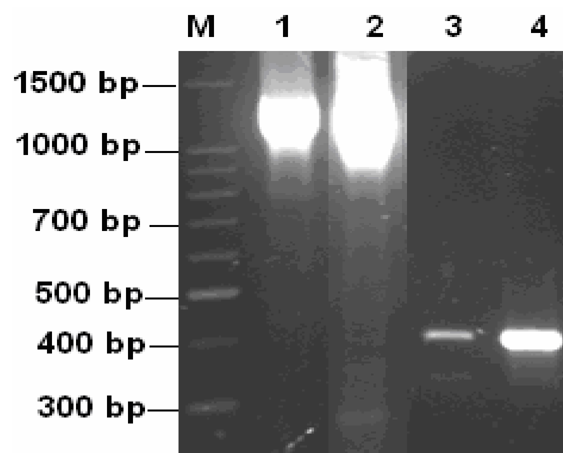

Figure 1. Representative ethidium bromide-stained 1.5% agarose gel analysis of PCR-amplified adhesion genes sequences. M, 100 bp marker (Promega, USA); Lanes 1 and 3, *icaA* and *icaD* positive PCR control (*S. aureus* CETC 975), respectively; Lane 2 and 4, *icaA* and *icaD* positive clinical isolate, respectively.

3. Results

The prevalence of intercellular adhesion and toxin genes is presented in Table 2. Both *icaA* and *icaD* were detected in 91% of the clinical *S. aureus* isolates. The positive isolates produced 1315 bp and 381 bp PCR products, for the *icaA* and *icaD* genes, respectively (Figure 1). The presence of the *icaA* and *icaD* genes was positively associated with the slime production (r = 1). These genes were detected in all isolates that showed black colonies on CRA.

The prevalence of *tst* gene in the clinical isolates was 43%. The *tst* positive isolates produced 180 bp PCR products (Figure 2). Only 2% and 1% of the clinical isolates were positive for the *eta* and *etb* genes, respectively. The *eta* and *etb* positive clinical isolates produced 190 bp and 612 bp PCR products, respectively (Figure 2).

Table 1. Primers, amplification conditions, primer concentration and anticipated sizes of PCR products for tested genes.

Gene	Primer	Oligonucleotide sequence 5'→3'	Size of amplified product (bp)	Primer concentration	Amplification conditions* and No. of cycles	Reference Strain	References
icaA	ICAAF ICAAR	F: 5'-CCTAAC TAA CGA AAG GTA G-3') R: 5'-AAG ATATAG CGA TAA GTG C-3'	1315	1 µM	45sec, 92°C; 45sec, 49°C; 1 min, 72°C; 35 cycle	CECT975	Vasudevan et al., 2003
icaD	ICADF ICADR	F: 5'-AAACGTAAGAGAGGTGG-3' R: 5'-GGCAATATGATCAAGATAC-3'	381	1 µM		CECT975	
tst	TST-1 TST-2	F: 5'-TTCACTATTTGTAAAAGTGTCAGACCCACT-3' R: 5'-TACTAATGAATTTTTTTATCGTAAGCCCTT-3	180	20pmol	30sec, 94°C; 30sec, 55°C; 1 min, 72°C; 35 cycle	NCTC 11963	Jarraud et al., 2002
eta	mpETA-1 mpETA-3	F: 5'-ACTGTAGGAGCTAGTGCATTTGT-3 R: 5'-TGGATACTTTTGTCTATCTTTTTCATCAAC-3'	190	20pmol		E-1	
etb	mpETB-1 mpETB-2	F: 5'-CAGATAAAGAGCTTTATACACACATTAC-3' R: 5'-AGTGAACTTATCTTTCTATTGAAAAACACTC-3	612	20pmol		TY4	

*Initial denaturation at 94°C for 2 min at the beginning of PCR; Final extension at 72° C for 10 min at the end of the cycles.

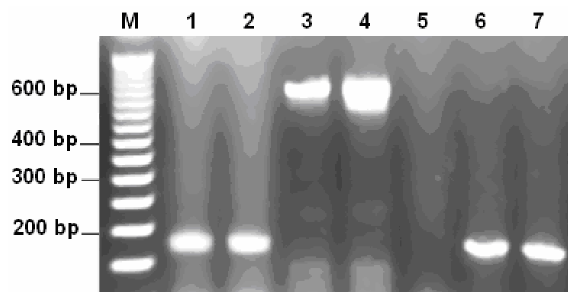

Figure 2. Representative ethidium bromide-stained 1.5% agarose gel analysis of PCR-amplified toxin genes sequences. M, 50 bp marker (Promega, USA); Lane 1, eta positive PCR control (E-1); Lane 2, eta positive clinical isolate; Lane 3, etb positive PCR control (TY4); Lane 4, etb positive clinical isolate; Lane 6, tst positive PCR control (S. aureus NCTC 11963); Lanes 7, tst positive clinical isolates; Lane 5, negative PCR blank.

Table 2. The prevalence and coexistence of adhesion (icaA, icaD) and toxin (tst, eta, etb) genes in 100 clinical S. aureus isolates.

Genes	No. (%) of positive isolates
Single possession of genes:	
icaA	91 (91)
icaD	91 (91)
tst	43 (43)
eta	2 (2)
etb	1 (1)
Multiple possession of genes:	
icaA + *icaD*	91 (91)
icaA + *icaD* + *eta*	1 (1)
icaA + *icaD* + *tst*	42 (42)

The coexistence of the studied adhesion and toxin genes in the *S. aureus* isolates is shown in Table 2. All isolates harboring the *icaA* gene were also harboring *icaD* gene. Only one *eta* containing isolate was positive for *icaA* and *icaD* genes. Most of the *tst* containing isolates (42/43) were positive for *icaA* and *icaD* genes. None of the isolates that contained *eta* or *etb* were positive for *tst* gene and the *etb* harboring isolates did not show any of the *eta*, *icaA* or *icaD* genes (Table 2).

Gene combination with the previously studied enterotoxin genes (*sea*, *sec*, *seg*, *seh* and *sei*) of the same 100 isolates (El-Huneidi *et al*., 2006; Naffa *et al*., 2006) was observed in 47 clinical isolates which fall into three groups (Table 3). These groups differ in the profile of gene combinations. Group I includes one *eta* + *sea* containing isolate which lacks *icaA*, *icaD* and *tst* genes. Group II includes 25 (53.2%) isolates lacking *tst* gene but harboring *icaA* + *icaD* genes in 11 different combinations with the enterotoxin genes. Group III includes 21(44.7%) isolates harboring *icaA* + *icaD* + *tst* in 7 different combinations with the enterotoxin genes. Interestingly, 20 isolates of Group III (gene combination number 1-6) have the corresponding gene combination profiles of 20 isolates of group II (gene combination number 1-6) in addition to the *tst* gene. Only one isolate of Group III (gene combination number 7) does not have corresponding gene combination in Group II isolates. The most frequent (17%) gene combination profile was detected in group II isolates. There was no significant difference ($p > 0.05$) between the prevalence of this combination and the corresponding one (8.5 %) in group III isolates harboring the additional *tst* gene. The second frequent (12.8%) combination profile was detected in group III isolates. There was no significant difference ($p > 0.05$) between the prevalence of this combination and its corresponding one in group II isolates, which represents the third frequent (10.6%) combination. Similarly, there was no significant difference ($p > 0.05$) between the prevalence of other combination profiles in group II isolates and their corresponding ones in group III isolates. A limited number of group II isolates (gene combination number 7-11) do not have corresponding combination profile in Group III isolates. On the other hand, only one isolate in group III harboring *icaA* + *icaD* + *tst* + enterotoxin genes and does not have corresponding combination profile in Group II isolates.

4. Discussion

This study presents an analysis of some virulence determinants in 100 clinical *S. aureus* isolates. It demonstrates the existence of the chromosomal *icaA* and *icaD* genes in 91% of *S. aureus* isolates. The coexistence

of these genes is correlated with slime production on Congo red agar in 91 % of *S. aureus* isolates. The high prevalence of these genes is consistent with that reported by other investigators (Montanaro *et al*., 1999; Ando *et al*., 2004; Peacock *et al*., 2002; Ando *et al*., 2004) and emphasizes the implication of these genes as virulence markers in biofilm formation. Both *icaA* and *icaD* genes are involved in formation of the capsular polysaccharide that also allows the bacteria to escape the immune system (Cramton *et al*., 1999; Arciola *et al*., 2001a, b; Vasudevan *et al*., 2003; Ando *et al*., 2004). However, the failure in slime production by 9% of *S. aureus* isolates might not affect formation of the biofilm, if yet unidentified *ica*-independent mechanisms of biofilm formation or a virulence determinant analogous to Biofilms Associated Protein (BAP) exists in these isolates. BAP is a surface protein and was detected in 5% of bovine mastitis *S. aureus* isolates (Cucarella *et al*., 2004). This protein promotes both primary attachment to inert surfaces and intercellular adhesion and is sufficient to induce biofilm production on abiotic surfaces when the *ica* locus product is absent (Cucarella *et al*., 2004).

In this report, few clinical isolates were harboring the exfoliative toxins genes (*eta* and *etb*). Therefore, screening for these genes in larger samples is necessary to give better information about the prevalence of such genes. The low frequency of these genes is in agreement with the results of other investigators, where Mehrotra *et al*. (2000) found that none of the 107 Canadian nasal isolates were positive for both *eta* and *etb* while the three clinical isolates were positive for both *eta* and *etb*. In Germany, 0.5% of the blood isolates were *eta* positive and none were *etb* positive, while 1.9% and 1% of the nasal isolates were *eta* and *etb* positive, respectively (Becker *et al*., 2003). The limited distribution of these genes suggested that certain isolates acquired the genes by horizontal gene transfer through plasmids or temperate bacteriophages. The *etb* gene is located on large plasmids while *eta* gene is carried on the genome of a temperate phage integrated in the *S. aureus* genome (Yamaguchi *et al.*, 2001).

Results of this study showed a high prevalence (43%) of *tst* in the clinical isolates (Table 2) which could be correlated with the transfer of this gene at high frequency (Moore and Lindsay, 2001). Similar prevalence (40%) of *tst* was reported in methicillin-susceptible *S. aureus* (MSSA) at the University Hospital in Magdeburg, Germany (Layer *et al*., 2006). Higher prevalence (72.5%) was detected in Japanese clinical isolates (Ando *et al*., 2004). Lower prevalence was detected in German blood (18.3%) and nasal isolates (22.4%) (Becker *et al*., 2003), Polish nasal isolates (10.5%) (Bania *et al*., 2006), German animal isolates (15.5%) (Akineden *et al*., 2001), and other animal isolates (26.7%) from different countries (Smyth *et a*l., 2005). Most (42/43) Jordanian *tst* positive isolates are slime formers (Table 2) and 50% (21/42) of these isolates contain various combinations of enterotoxin genes (Table 3). The profile of gene combination (number 1-6) in 20 *tst* positive isolates (group III, Table 3) is analogous to that detected in other 20 isolates of group II lacking *tst* gene The presence and absence of *tst* in these groups of isolates can be attributed to the presence of *tst* on a mobile genetic element called staphylococcal pathogenicity islands (SaPI1 and SaPI2) (Schmidt and Hensel, 2004). Loss or acquisition of these islands could be the mechanism that contributes to the appearance of these groups of isolates. Transduction of SaPI1 and SaPI2 by helper phages was demonstrated (Lindsay *et al*., 1998; Ruzin *et al.*, 2001). In the absence of these helper phages, these islands remain stably integrated in the chromosome (Schmidt and Hensel, 2004). A pathogenicity island (SaPIbov) related to SaPI1 was identified in bovine isolates of *S. aureus* (Fitzgerald *et al*., 2001). SaPIbov harbors *tst*, *sec* and *sel* genes (Schmidt and Hensel, 2004). The co-existence of the *tst* and *sec* genes in animal isolates including bovine has been reported (Akineden *et al*., 2001; Fitzgerald *et al*., 2001; Smyth *et al*., 2005). This contrasts with findings of rare co-existence of *tst* and *sec* genes in 3 clinical isolates of Group III in the present study and in other studies (Peacock *et al*., 2002; Becker *et al*., 2003; Bania *et al*., 2006; Layer *et al*., 2006) and confirms that SaPI1 and SaPI2 lack *sec*.

Several investigators (Peacock *et al*., 2002; Becker *et al*., 2003; Layer *et al*., 2006) suggested that a number of bacterial determinants act in combination during the infective process. The presence of various gene combinations in 46 slime forming isolates in this study (groups II and III, Table 3) supports some sort of association between pathogenicity and colonization genes of *S. aureus*. The predominant gene combinations in these isolates include *seg* + *sei* or *seg*. These genes belong to the enterotoxin gene cluster (*egc*) that was identified by Jarraud *et al* (2001). This finding is consistent with that reported in other countries for human (Becker *et al*., 2003; Bania *et al.*, 2006; Layer *et al*., 2006) and animal strains of *S. aureus* (Akineden *et al.*, 2001; Smyth *et al*., 2005). The dominancy of the *egc* cluster genes in human and animal isolates suggests a potential role of these superantigens in different infections caused by *S. aureus*.

It is important to mention that PCR is able to demonstrate the existence of genes in *S. aureus* isolates but it does not prove the production of the proteins encoded by these genes. Therefore, bioassay or immunological methods must be used to demonstrate the ability of the Jordanian isolates to produce the toxin and the adhesion proteins.

In conclusion, this study has demonstrated the variable presence of *icaA* and *icaD*, *tst, eta,* and *etb* genes in the clinical isolates of *S. aureus*. These genes also coexist in different combinations with the previously detected enterotoxin genes (Naffa *et al*., 2006; El-Huneidi *et al*., 2006), supporting the notion that these genes act in combination during infection. This data may help in providing a guideline for the control of colonization and the spread of these isolates in the hospital environment and community.

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Our thanks are due to Dr. Motoyuki Sugai (Graduate School of Biomedical Sciences, Dept. of Bacteriology, Hiroshima University, Hiroshima, Japan) for providing us with the E-1 (*eta* positive) and TY4 (*etb* positive) *S. aureus* strains.

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