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

Ala'a M. Bawadi, Salwa Bdour and Adel Mahasneh*

Department of Biological Sciences, Faculty of Science, University of Jordan, Amman Jordan

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 icaA+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.

Keywords: Staphylococcus Aureus; Adhesion Genes; Toxin Genes; Jordan.

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 with UDP-N-acetylglucosamine (Arciola et al., 2001a).

*Corresponding author. amahasneh@ju.edu.jo.
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 (Nafta 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](image-url)

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 CECT 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Chromosomal genes</th>
<th>Size of amplification product (bp)</th>
<th>Primers concentration</th>
<th>Amplification condition</th>
<th>Reference State</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>icaA</td>
<td>GAAAY</td>
<td>GAAA</td>
<td>icaA, etb</td>
<td>315</td>
<td>1 µM</td>
<td>4 sec, 94°C, 40 sec, 1 min, 72°C × 30 cycle</td>
<td>CECT905</td>
<td>Haller, 2008</td>
</tr>
<tr>
<td>icaD</td>
<td>GAAAY</td>
<td>GAAA</td>
<td>etb, icaA</td>
<td>381</td>
<td>1 µM</td>
<td>4 sec, 94°C, 40 sec, 1 min, 72°C × 30 cycle</td>
<td>CECT905</td>
<td>Haller, 2008</td>
</tr>
<tr>
<td>tst</td>
<td>TST1</td>
<td>TST2</td>
<td>etb, icaA, etb</td>
<td>180</td>
<td>20 µmol</td>
<td>4 sec, 94°C, 2 sec, 95°C, 1 min, 72°C × 30 cycle</td>
<td>NCTC 11963</td>
<td>El-Huneidi, 2004</td>
</tr>
<tr>
<td>eta</td>
<td>ETA1</td>
<td>ETA2</td>
<td>eta, icaA, etb</td>
<td>190</td>
<td>20 µmol</td>
<td>4 sec, 94°C, 2 sec, 95°C, 1 min, 72°C × 30 cycle</td>
<td>NCTC 11963</td>
<td>El-Huneidi, 2004</td>
</tr>
<tr>
<td>etb</td>
<td>ETB1</td>
<td>ETB2</td>
<td>eta, icaA, etb</td>
<td>612</td>
<td>20 µmol</td>
<td>4 sec, 94°C, 2 sec, 95°C, 1 min, 72°C × 30 cycle</td>
<td>NCTC 11963</td>
<td>El-Huneidi, 2004</td>
</tr>
</tbody>
</table>

*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.

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

<table>
<thead>
<tr>
<th>Genes</th>
<th>No. (%) of positive isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single possession of genes:</td>
<td></td>
</tr>
<tr>
<td>icaA</td>
<td>91 (91)</td>
</tr>
<tr>
<td>icaD</td>
<td>91 (91)</td>
</tr>
<tr>
<td>tst</td>
<td>43 (43)</td>
</tr>
<tr>
<td>eta</td>
<td>2 (2)</td>
</tr>
<tr>
<td>etb</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Multiple possession of genes:</td>
<td></td>
</tr>
<tr>
<td>icaA + icaD</td>
<td>91 (91)</td>
</tr>
<tr>
<td>icaA + icaD + eta</td>
<td>1 (1)</td>
</tr>
<tr>
<td>icaA + icaD + tst</td>
<td>42 (42)</td>
</tr>
</tbody>
</table>

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 importance 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 Mehrrota 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%) (Akinenden et al., 2001), and other animal isolates (26.7%) from different countries (Smyth et al., 2005). Most (42/43) Jordanian *tst* positive isolates are slime formers (Table 2) and 50% (21/42) of these isolates contain various combinations of enteroxin 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 (SaPIblov) related to SaPI1 was identified in bovine isolates of *S. aureus* (Fitzgerald et al., 2001). SaPIblov 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 (Akinenden 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 enteroxin 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* (Akinenden 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 enteroxin 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.

**Acknowledgment**

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 TV4 (*etb* positive) *S. aureus* strains.
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