Enterotoxicity and genetic variation among clinical Staphylococcus aureus isolates in Jordan

Randa G. Naffa, 1 Salwa M. Bdour, 1 Hussein M. Migdadi2 and Asem A. Shehabi3

1, 3Department of Biological Sciences, Faculty of Science1 and Department of Pathology and Microbiology, Faculty of Medicine3, University of Jordan, Amman, Jordan
2National Center for Agricultural Research and Technology Transfer, Amman, Jordan

A total of 100 Jordanian clinical Staphylococcus aureus isolates was analysed for the presence of the enterotoxin genes sea, seb, sec, sed and see using multiplex PCR. Twenty-three isolates (23 %) were potentially enterotoxigenic. The prevalence of sea, sec and sea plus sec among the total clinical isolates was 15, 4 and 4 %, respectively. None of the isolates harboured sed, seb or see genes. S. aureus isolates were subjected to DNA fingerprinting by randomly amplified polymorphic DNA (RAPD) analysis to test whether isolates harbouring the toxin genes were genetically clustered. A total of 13 genotypes was identified at a 47 % similarity level. Genotypes I and V accounted for the largest number of enterotoxigenic isolates (19 %). This study has demonstrated the genetic diversity of Jordanian clinical S. aureus isolates and shown that the presence of the toxin genes is not genotype specific.

INTRODUCTION

Staphylococcus aureus produces a variety of extracellular toxins and virulence factors that contribute to its pathogenic potential. Some S. aureus strains produce pyrogenic exotoxins, such as staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin-1 (Sharma et al., 2000). SEs are a group of single-chain, low-molecular-mass proteins that are similar in composition and biological activity but differ in antigenicity (Fueyo et al., 2001). Several serologically distinct SEs have been recognized, comprising the classical SEs (SEA to SEE) (Dinges et al., 2000; Becker et al., 2003) and the newly described SEs (SEG to SER and SEU) (Su & Wong, 1995; Jarraud et al., 2001; Letertre et al., 2003).

The enterotoxins of S. aureus can be detected by their biological activity, by immunoassays and by PCR (McLauchlin et al., 2000). The prevalence of enterotoxigenic clinical S. aureus isolates has been reported in different countries by many investigators (Mehrotra et al., 2000; Fueyo et al., 2001; Becker et al., 2003). There are no published reports on the prevalence of these isolates in Jordanian S. aureus isolates.

Accurate and rapid typing of S. aureus strains is crucial to the control of infectious strains. Numerous typing methods have been described (Frénay et al., 1994; Kluytmans et al., 1995; Yoshida et al., 1997; van Leeuwen et al., 2003); among these is randomly amplified polymorphic DNA (RAPD) analysis, which has been found to be a simple, rapid and effective method for genotyping of S. aureus (Tambic et al., 1997).

The purpose of this study was to investigate (i) the prevalence of the classical enterotoxin genes in Jordanian clinical isolates of S. aureus, (ii) the genetic variation among these isolates using RAPD analysis and (iii) the genotype specificity of the classical enterotoxin genes.

METHODS

Bacterial strains. This study used 100 clinical S. aureus isolates that were collected and identified by biochemical tests during a previous study (Al-Zu’bi et al., 2004). These isolates were obtained from various clinical specimens submitted to the microbiology laboratory of Jordan University Hospital, Amman, Jordan. In all assays, the following enterotoxigenic S. aureus reference strains were used as positive controls: CECT 976 (SEA positive), CECT 4459 (SEB positive), CECT 4465 (SEC positive) and CECT 4466 (SED positive) [kindly provided by the Spanish Type Culture Collection (CECT)], while the DNA of ATCC 27664 (SEE positive) was kindly provided by Dr Karsten Becker (Institute of Medical Microbiology, University Hospital of Münster, Germany).

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, except that the pelleted bacterial cells were first treated with 60 μl lysozyme (10 mg ml−1; ICN Biomedicals) and 9 μl lysostaphin (1 mg ml−1; Sigma) for 1 h at 37 °C. The preparations were analysed on a 0.7 % agarose gel and the quantity and quality of DNA were determined spectrophotometrically (Sambrook et al., 1989). The amount of DNA was adjusted to the required concentration for each PCR for detection of enterotoxin genes and for genotyping of S. aureus isolates.

Abbreviations: RAPD, randomly amplified polymorphic DNA; SE, staphylococcal enterotoxin.
Detection of enterotoxin genes by PCR. Genomic DNA (50–100 ng) of S. aureus strains was amplified in two sets of multiplex PCR as reported by Rosec & Gigaud (2002). Set A contained 3 ng of each sea, sed and see primer μl⁻¹, while set B contained 3 ng of each seb and sec primer μl⁻¹. 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. A positive PCR control containing DNA of the reference strains and a negative PCR blank control with nuclease-free water instead of genomic DNA were included with each set of five reactions.

RAPD-PCR typing. Four random-sequence primers were used in four separate RAPD-PCR tests for typing of S. aureus isolates. Primers S (5′-TCACGATGCA-3′) and C (5′-AGGGAACGAG-3′) (Fueyo et al., 2001) and primer OPA13 (5′-CAGCACCCGAC-3′) (Byun et al., 1997) were selected because they previously showed good discriminatory power in RAPD analysis of S. aureus (Fueyo et al., 2001; Byun et al., 1997). Primer OPA09 (5′-GGGTAACGCC-3′) (Operon Technologies) was used after optimization of PCR conditions for ultimate discriminatory power.

RAPD-PCR with 0·5 μM primer S or C and 100 ng genomic DNA was performed according to Fueyo et al. (2001), and with 0·1 μM primer OPA13 and 50 ng DNA was performed according to Byun et al. (1997). After optimization of PCR components and conditions, RAPD-PCR with OPA09 was carried out in a 25 μl reaction mixture containing 12·5 μl PCR Master Mix (Promega), 10 pmol primer, 3 U Taq DNA polymerase, 3·5 mM MgCl₂, 10 ng DNA template and nuclease-free water. Amplification conditions consisted of denaturation at 94 °C for 60 s and 35 cycles of denaturation at 94 °C for 35 s, annealing at 33 °C for 30 s and extension at 72 °C for 65 s. PCR products were detected in 1 % agarose gel.

Computer analysis of RAPD data. RAPD banding patterns of the 100 isolates of S. aureus were examined and the size of bands was estimated by using the standard curve for DNA marker fragments in each gel (Sambrook et al., 1989). Bands were scored, with the data coded as a factor of 1 or 0, representing the presence or absence of bands, respectively. Banding patterns of the summed results for the four primers were used for cluster analysis in the RAPD assay. A similarity matrix among S. aureus isolates was produced by using the Jaccard coefficient (Jaccard, 1908) and a dendrogram showing the genetic relatedness among the isolates was constructed from the resulting data using spss version 10. The cut-off for the dendrogram was selected based on the average of mean similarity matrix.

Statistical analysis. A χ² 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 (Levin & Rubin, 1991).

RESULTS

Prevalence of the enterotoxin genes sea, seb, sec, sed and see

A total of 23 clinical isolates (of 100) of S. aureus were harbouring sea and sec genes, either singly or in combination. Fifteen of the total isolates were positive for the sea gene, four were positive for the sec gene and four were positive for both sea and sec genes. None of the isolates harboured sed, seb or see genes. The sizes of the amplicons...
obtained for the enterotoxin genes corresponded to the anticipated sizes reported by Rosec & Gigaud (2002) (Fig. 1).

**RAPD analysis**

RAPD analysis with the four primers showed consistently different banding patterns with reproducible polymorphic bands of variable size and number. Amplification with primer S resulted in 27 polymorphic bands with nine RAPD patterns. Primer C produced 19 polymorphic bands with 16 RAPD patterns, while primer OPA09 produced 24 polymorphic bands with 11 RAPD patterns. Primer OPA13 generated 30 polymorphic bands with 20 RAPD patterns (Fig. 2). All 100 polymorphic bands produced were used for cluster analysis. The genetic similarity indices based on Jaccard coefficients ranged from 0.1 to 1.0. This wide range of similarity indices indicated a high level of DNA polymorphism among the *S. aureus* isolates. The average mean similarity index was 0.47, indicating that the isolates shared 47% of their RAPD fragments.

**Genetic diversity of *S. aureus* isolates**

Fig. 3 shows a dendrogram constructed on the basis of similarity index among *S. aureus* isolates using the four RAPD primers. A 47% similarity cut-off value gave 13 major clusters (RAPD genotypes) (I–XIII) and seven subclusters (RAPD subtypes) including genetically related isolates. The majority of *S. aureus* isolates (43) belonged to genotype I (range of similarity 0.46–1.00). The others were distributed as follows: 26 isolates in genotype VI (range of similarity 0.30–1.00), 17 isolates in genotype V (range of similarity 0.41–0.85), three isolates in genotype X (range of similarity 0.6–0.8), two isolates in genotype VII (similarity level 0.74) and two isolates in genotype VIII (similarity level 0.6). Seven isolates formed single clusters (genotypes) (II, III, IV, IX, XI, XII and XIII). On the other hand, genotype VI included three subtypes: VIa at a 0.63 similarity level and VIb and VIc at a 0.55 similarity level. Genotype V included four subtypes: Va, Vb and Vc at a 0.60 similarity level and Vd at a 0.56 similarity level.

**Distribution of enterotoxin genes among *S. aureus* genotypes**

Table 1 shows the distribution of the enterotoxin genes among *S. aureus* genotypes. The *sea* gene was carried either singly or in combination with the *sec* gene by some isolates of genotypes I, V (subtypes Vb, Vc and Vd), VI, IX and X. The *sec* gene was harboured by some isolates of genotypes V (subtypes Vb and Vd), VI (subtype VIa) and X. On the other hand, isolates of the single genotypes (II, III, IV, IX, XII and XIII), genotypes VII and VIII and subtypes Va, Vlb and Vlc were negative for the toxin genes.

A χ² 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.
Table 1. Distribution of classical enterotoxin genes among Jordanian *S. aureus* genotypes

<table>
<thead>
<tr>
<th>Cluster (RAPD genotype)</th>
<th>Number of isolates</th>
<th>Subtype (n)</th>
<th>SE gene(s) detected (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>43</td>
<td>sea (11)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>1</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>1</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>1</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>17</td>
<td>Va (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vb (5)</td>
<td>sea + sec (1), sec (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vc (8)</td>
<td>sea (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vd (2)</td>
<td>sea + sec (1), sec (1)</td>
</tr>
<tr>
<td>VI</td>
<td>26</td>
<td>Vla (6)</td>
<td>sea + sec (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vlb (16)</td>
<td>NE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vlc (4)</td>
<td>NE</td>
</tr>
<tr>
<td>VII</td>
<td>2</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>VIII</td>
<td>2</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>IX</td>
<td>1</td>
<td>sea (1)</td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>3</td>
<td>sea + sec (1), sec (1)</td>
<td></td>
</tr>
<tr>
<td>XI</td>
<td>1</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>XII</td>
<td>1</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>XIII</td>
<td>1</td>
<td>NE</td>
<td></td>
</tr>
</tbody>
</table>

NE, Non-enterotoxigenic.

**DISCUSSION**

The prevalence of the five classical enterotoxin genes in 100 clinical *S. aureus* isolates and correlation of their prevalence with RAPD genotypes were studied. Up to 23% of the isolates harboured either the *sea* or the *sec* gene or both. A higher prevalence rate was reported for the classical *sea–sec* genes in German multicentre clinical (43%) and nasal (39.5%) isolates (Becker et al., 2003) and in isolates collected from Japanese patients with food poisoning (76%) (Omoe et al., 2002). This variation in prevalence is probably due to geographical differences, which may be further affected by the different ecological origins of the isolated strains (food, humans and animals) (Mehrotra et al., 2000; Fueyo et al., 2001; Becker et al., 2003).

In the present study, the *sea* gene dominated over the *sec* gene. Similar results have been reported in Germany (Becker et al., 2003) and Canada (Mehrotra et al., 2000) among isolates of human origin. However, other investigators have reported that the *sec* gene is the most frequent among strains of bovine origin in Europe and the USA (Valle et al., 1990; Sharma et al., 2000; Larsen et al., 2002).

It is important to recognize that the presence of the *sea* and *sec* genes in Jordanian *S. aureus* isolates does not necessarily indicate the ability of these isolates to produce intact and biologically active toxin or to produce sufficient toxin to induce disease. Detection of these genes by PCR may indicate their potential to cause disease. Fueyo et al. (2001) reported that the detection of the classical enterotoxin genes in Spanish *S. aureus* isolates by PCR was fully concurrent with the production of toxin by these isolates. In contrast, Sharma et al. (2000) reported that toxin genes were detected in toxin-non-producing strains of *S. aureus*, due either to low-level production of enterotoxin or to mutations in the coding region or in a regulatory region(s) (Sharma et al., 2000).

Typing by RAPD revealed that the 100 clinical *S. aureus* isolates were genetically diverse and comprised a heterogeneous population with 13 genotypes at a 47% similarity level. Genotype I appeared to be predominant. The presence of *sea* and *sec* genes was not genotype specific. Genotype I included only *sea*-positive isolates (Table 1), while genotype V included a great diversity of *sea- and sec*-positive isolates. More importantly, pairs of identical isolates with a similarity index of 1.00 (e.g. pairs of isolates numbered 11 and 65, 38 and 63, and 67 and 68 of genotype I; Fig. 3) included *sea- and sec*-positive and -negative isolates. This result provides evidence that loss and/or acquisition of enterotoxin genes occurs within the same genetic background and that the presence of these genes is not genotype specific. In agreement with our results, Fueyo et al. (2001) 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. (2002) showed that there was no association between RAPD genotypes and the presence of toxin genes.

Horizontal movement of *sea* or *sec* genes could occur among isolates of different genotypes. This movement appeared to be restricted to five genotypes (I, V, VI, IX and X) (Table 1) and could be extended to other genotypes and involve isolates negative for the toxin genes. The horizontal transfer of enterotoxin genes is mostly mediated by generalized transduction (Moore & Lindsay, 2001). The fact that genotypes I and V included most *sea- and sec*-positive *S. aureus* isolates may indicate their occurrence as epidemic isolates in the Jordanian community and hospitals.

In conclusion, the present investigation demonstrated that the *sea* gene was the predominant enterotoxin gene in these genetically diverse Jordanian clinical isolates and that the presence of these genes was not genotype specific.

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REFERENCES


