DETECTION OF ENTEROTOXIGENIC
STAPHYLOCCUS AUREUS STRAINS
USING A COMMERCIAL ELISA AND MULTIPLEX-PCR

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Abstract

A commercial ELISA and multiplex-PCR were used to determine the enterotoxigenicity of 99 S. aureus strains. In 36 strains the genes of enterotoxins SEA to SEE were detected. Out of these, 32 strains were found to be enterotoxin producers. In the remaining 4 strains the enterotoxin was not detected. These 4 strains were tested by RT-PCR for SE mRNA presence. In the case of sea- and sec-harbouring strains, the presence or absence of an RT-PCR product was consistent with the presence and absence of an ELISA signal, respectively. In the case of strains carrying the sed gene, a similar correlation could not be observed. The presence of sed RT-PCR product was found in all the strains regardless of their ability to produce SED.

Key words: Staphylococcus aureus, enterotoxin, ELISA, PCR.

Staphylococcus aureus is considered to be one of the major pathogens causing outbreaks of food poisoning, being outnumbered only by Salmonella sp. (1). Staphylococcal food poisoning (SFP) develops following ingestion of products that contain staphylococcal enterotoxin (SE) and is manifested by vomiting, frequently accompanied by gastroenteritis. The concentration of SE necessary to cause intoxication is very small, ranging from 0.5 to 1.0 ng/ml and depends on the susceptibility of the individual (6). Enterotoxins are relatively thermostable and resistant to inactivation by gastrointestinal enzymes. Their heat stability seems to depend on the environmental factors of the medium the toxin is in, especially the water activity (a_w) and pH (3). According to the standard nomenclature, only toxins that induce emesis after oral administration in a primate model can be designated as SEs. Other related toxins that either lack emetic properties or have not yet been tested should be designated as staphylococcal enterotoxin-like superantigens (SEls) (11). The enterotoxin family now contains 18 toxins. The SE family is divided into the classical enterotoxins SEA to SEE and a group of recently discovered toxins that comprise both true SEs, namely SEG, SEH, and SEI, as well as SEls designated as SEIJ to SEIP and SEIU (3, 10).

SEA and SED, the most frequently recovered from food involved in food poisoning, are thought to be the major cause of SFP (3, 10). It is estimated that about 5% of food poisoning cases in which none of the classical enterotoxins were detected can, however, be attributed to new enterotoxins (9, 10, 15). Of these, SEH was the most commonly reported as the potential cause of SFP (9, 12, 17). Of the other new SEs only SEI, SEG, and SEIP were shown to elicit emesis in various models, but their involvement in food poisoning still remains unclear (12, 13).

There are a number of methods to detect staphylococcal enterotoxigenicity including biological, and immunological assays, and DNA amplification in PCR. ELISA can demonstrate enterotoxin presence directly in food, allowing the detection of nanogram amounts of the toxin in 1 g of sample. PCR-based methods showing the presence of enterotoxin genes demonstrate potential enterotoxigenicity of S. aureus. In this study a commercial ELISA and a multiplex-PCR test for the detection of enterotoxigenic S. aureus strains were compared.

Material and Methods

Bacterial strains. Fifty S. aureus isolates were derived from raw-minced pork and turkey meat (4). Forty-nine S. aureus cultures were isolated at the
Laboratory of Bacteriology and Immunology, Korczak Pediatric Center of Lower Silesia, Wroclaw, Poland, from faecal swabs of ambulatory patients suffering from diarrhoea. Three enterotoxigenic reference strains, CCM5757 (SEB), FR1151m (SED) and FR1913 (SEA, SEC, SEE) were used as positive controls. These strains were kindly provided by Prof. Gerard Lina from the Centre National de Référence des Toxémies Staphyloccociques, Faculté de Médecine, Lyon, France.

Preparation of bacterial DNA. Two milliliters of a bacterial cell suspension from an overnight culture grown in brain-heart infusion were centrifuged for 5 min at 12 000 × g and suspended in 100 µl of 100 mM Tris-HCl buffer, pH 7.4, containing 10 µg of lyostaphin (Sigma-Aldrich, Poland). After 30 min of incubation at 37°C, 10 µl of 10% SDS were added and the sample was incubated for another 30 min at 37°C. Two hundred microlitres of 5 M guanidine hydrochloride were added and the sample was mixed by vortexing and incubated at room temperature for 10 min. The DNA was extracted by phenol and chloroform, ethanol-precipitated, and dissolved in water.

Detection of enterotoxin genes by PCR. We used the set of primers described by Sharma et al. (16), and designed for a multiplex PCR to detect the five enterotoxin genes from SEA to SEE, using one universal, forward primer and five gene-specific reverse primers for each of the enterotoxin genes. The PCR was performed in a total volume of 25 µl. The reaction buffer contained 50 mM KCl, 10 mM Tris-HCl, 4 mM MgCl₂, 0.2 mM of each dNTP, 30 pmole of each primer (Institute of Biochemistry and Biophysics, Warsaw, Poland), 1 µl of DNA solution, and 1 U of Taq DNA polymerase (Fermentas, Lithuania). Thirty-five cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 45 s were performed using the T3 thermal cycler (Biometra, Germany). A 10 µl aliquot of PCR product was resolved on 2% agarose gel at 100 V and documented with a camera system (Velbr-Lourmat, France).

Detection of staphylococcal enterotoxins by ELISA. S. aureus was detected in Staphylococcus Growth Medium (TECRA, Australia) for 20 h at 37°C. The cultures were centrifuged for 10 min at 3 000 × g and the supernatant was tested with a TECRA SET VIA kit (Staphylococcal Enterotoxin Visual Immunoassay) for bulk detection of the enterotoxins SEA, SEB, SEC, SEC, SEC, SEC, and SEE, and alternatively with a TECRA SET ID kit (Staphylococcal Enterotoxin Identification), for identification of the specific enterotoxin type. The tests were performed according to the manufacturer’s instructions. The absorbance of samples was read at 414 nm using a Spectra plate-reader (SLT Labinstruments, Germany). The samples with A414 below 0.05 were assigned as negative, whereas the samples with A414 above 1 were assigned as positive.

Reverse transcription of SE mRNA. Two ml of S. aureus culture grown in Staphylococcus Growth Medium to a stationary phase (OD₅₆₀ = 6) were centrifuged at 12 000 × g and suspended in 100 µl of 100 mM Tris-HCl buffer, pH 7.4, containing 10 µg of lyostaphin; then, the mixture was incubated at 37°C for 20 min. The bacterial RNA was recovered using TRI reagent (Sigma-Aldrich) following the manufacturer’s instructions. Isopropanol-precipitated and ethanol-washed RNA was air-dried and suspended in 20 µl of water. One-microgram RNA samples, as assessed from A₂₆₀/A₂₃₀ readings, were treated with DNAsel I (Sigma-Aldrich), then heated at 70°C for 15 min for DNase inactivation, and subjected to reverse transcription (RT). Random hexamer primer (0.2 µg) was added to 11 µl of DNAsel-treated RNA sample, and the mixture was heated at 70°C for 5 min, then chilled on ice. The mixture was complemented with dNTP to a final concentration of 1 mM, Revert Aid reaction buffer, and 200 U of Revert Aid H Minus M-MuLV reverse transcriptase (Fermentas). RT was performed at 42°C for 1 h, then the mixture was heated at 70°C for 10 min. cDNA was subjected to PCR with the primers for SE genes, using the conditions as described above. The 23S rRNA coding gene was chosen as the reference gene. The primers were selected according to the published sequences of S. aureus 23S rDNA, 23S For: GTGTCAAGCGGCGAGTGT, and 23S-rev: TCCTAGTAAAGGACAGCTCCTC, giving a product of 433 bp. For 23S cDNA amplification, 20 PCR cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s were performed. Potential genomic DNA contamination was monitored by running the PCR with each DNAsel-treated RNA and the primers for 23S rDNA. Quantification of the PCR products was performed by analysing the gel images using the BioID program (Velbr-Lourmat). The amounts of RNA used for RT-PCR were normalized to the signal obtained from 23S cDNA amplification.

Results

Thirty-six of the 99 S. aureus strains were shown to harbour the enterotoxin sea-see: genes. sea was detected in 12 strains, seb in 3, sec in 8, and sed in 6 strains. The genotype sea/sec was detected in 4 strains, seb/sed in 1 strain, sea/sed in 1 strain, and the sea/sec/see genotype was detected in 1 strain (Table 1). The culture supernatants from all the tested strains were screened for enterotoxin SEA – SEE production using the TECRA SET VIA kit. Thirty-two strains, including all strains carrying single SE genes of sea and seb, were found to be enterotoxin producers. Out of the 6 strains containing only a sed gene, 3 produced SEC, while the remaining 3 were negative in both the TECRA SET VIA and TECRA SET ID kits. In the 8 strains carrying only an sec gene, 1 was found to be negative in ELISA. The strains harbouring multiple SE genes, i.e. sea/sed (1 strain), seb/sed (1 strain), and sea/sec/see (1 strain), assayed with the TECRA SET ID kit for determining the specific enterotoxin type, were positive for the respective enterotoxins. Among the 4 strains with the enterotoxin genotype sea/sec, 2 were shown to produce both SEA and SEC, 1 produced SEA only, whereas the last produced SEC only (Table 1). The 3 reference S. aureus strains were tested with the TECRA SET ID kit and were shown to produce the respective enterotoxins.
Six strains harbouring the SE genes whose products were not detected by TECRA SET VIA or by TECRA SET ID were tested with RT-PCR for SE mRNA presence. We found no sec band in RT-PCR performed on 1 sea/sec- and 1 sec-carrying strain, in which SEC was not detected by ELISA (Fig. 1, lanes 4 and 5). Alternatively, a sec band was obtained in RT-PCR performed on 1 control strain carrying the sec gene and producing SEC (Fig. 1, lane 6). A strain containing both sea and sec genes in which SEA was not detected by ELISA also produced no sea band in RT-PCR (Fig. 1, lane 3). This strain was shown to be an SEC-producer. Two control strains expressing SEA were shown to produce the sea band in RT-PCR (Fig. 1, lanes 1 and 2). RT-PCR was performed with all the 8 strains carrying the sed gene and on the FRI151m reference strain. All of them, including 6 strains shown to produce SED (Fig. 2 lanes 1, 2, 3, 4, 7, and 8) and 3 strains in which SED was not detected by ELISA (Fig. 2 lanes 5, 6, and 9), produced the sed RT-PCR product.

Table 1
Enterotoxin genotypes and production of enterotoxins by S. aureus strains

<table>
<thead>
<tr>
<th>Enterotoxin genotype</th>
<th>Number of S. aureus strains</th>
<th>Type of enterotoxin detected in ELISA</th>
<th>Number of S. aureus strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>sea</td>
<td>12</td>
<td>SEA</td>
<td>12</td>
</tr>
<tr>
<td>seb</td>
<td>3</td>
<td>SEB</td>
<td>3</td>
</tr>
<tr>
<td>sec</td>
<td>8</td>
<td>SEC</td>
<td>7</td>
</tr>
<tr>
<td>sed</td>
<td>6</td>
<td>SED</td>
<td>3</td>
</tr>
<tr>
<td>sea, sec</td>
<td></td>
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<td>2</td>
</tr>
<tr>
<td>sea, sed</td>
<td></td>
<td>SEA + SED</td>
<td>1</td>
</tr>
<tr>
<td>seb, sed</td>
<td></td>
<td>SEB + SED</td>
<td>1</td>
</tr>
<tr>
<td>sea, sec, see</td>
<td></td>
<td>SEA + SEC + SEE</td>
<td>1</td>
</tr>
</tbody>
</table>

Fig. 1. Agarose gel electrophoresis of sea, sec and 23S RT-PCR products. Lanes 1, 2, and 3 represent RT-PCR products from S. aureus strains harbouring the sea gene. Lanes 4, 5, and 6 represent RT-PCR products from S. aureus strains harbouring the sec gene. Insert below represents RT-PCR products from 23S gene used to normalize the amounts of sea and sec RNA in RT-PCR.
Fig. 2. Agarose gel electrophoresis of sed and 23S RT-PCR products. Consecutive lanes from 1 to 9 represent RT-PCR products from S. aureus strains harbouring the sed gene. Insert below represents RT-PCR products from 23S gene used to normalize the amounts of sed RNA in RT-PCR.

Discussion

In this study, the relationship between enterotoxin gene content and enterotoxin production was determined using a commercial ELISA kit. Thirty-two of 99 examined S. aureus strains were found to be enterotoxin producers, while 4 strains harbouring the enterotoxin genes were found to be not enterotoxigenic.

The detection of S. aureus strains sharing similar properties was previously reported. In the study of Atanassova et al. (2) performed with S. aureus strains isolated from fresh meat, 34.8% of the strains were harbouring one or more SE genes. The authors, using SET-RPLA (reversed passive agglutination) test, demonstrated that only 28.6% of the strains were enterotoxigenic. Sharma et al. (16), using the same method to assess enterotoxin production, did not find detectable levels of SEC in 2 S. aureus strains carrying the sec gene. Fueyo et al. (7), testing 269 S. aureus isolates, found that 4 isolates, which carried sea and tst genes, were TSST-1-negative. Also, Becker et al. (5), using a combination of multiplex-PCR and EIA (enzyme immunoassay) described an SEA-negative S. aureus strain containing the sea gene.

All our strains that harboured SE genes but were negative according to ELISA were tested by RT-PCR. In the case of strains harbouring sea and sec, the presence or absence of an RT-PCR product was consistent with the presence and absence of an ELISA signal, respectively. In the case of strains carrying the sed gene, a similar correlation could not be observed. The presence of sed RT-PCR product was found in all the strains, regardless of its ability to produce SED.

The expressions of the SEs investigated so far have been shown to be tightly regulated at the transcriptional level. Some of them, including SEB, SEC, and SED, produced mainly at late exponential or early stationary phase, are thought to be regulated by the agr (accessory gene regulator) operon (8, 14, 18). The agr was shown to act as a quorum-sensing system, being at the same time a global regulator of staphylococcal virulence gene expression. The key regulatory element, the RNA III protein, influences the transcription of many staphylococcal virulence factors, down-regulating some cell wall-associated proteins and simultaneously up-regulating the expression of secreted toxins (20). SEA is an example of a transcriptionally regulated SE, shown to be agr independent (19). The cause of discrepancy between sed RT-PCR product presence and SED production was not determined in this study. It is therefore not possible to state whether the regulation of SED production is also influenced at the level of translation. Although SED production has been shown to be regulated in an agr-dependent way, the possibility of translational regulation was not excluded in published reports (18). Moreover, the newly characterized enterotoxins SEG and SEI are likely regulated at the translational level, as their mRNA levels are not linked to the presence of protein (12). The difference between RT-PCR and ELISA results can also be explained by the low-level of SED production, i.e. below the threshold of immunoassay detection. If this is true, the concentration of toxin should be below 1 ng/ml, which is the lowest detection limit of the test used in this study. Another explanation might be that the non-productive sed genes were truncated or incomplete. This makes their PCR detection conditionally possible, but can strongly affect the production of mature toxin, leading to the inability of its detection in ELISA.
Another factor that differentiates SED from SEA and SEC is the type of genetic element carrying its genes and its location in the S. aureus genome. Prophage-encoded SEA and staphylococcal pathogenicity island-encoded SEB and SEC remain integrated in the S. aureus chromosome, while SED is plasmid-encoded. It is therefore possible that the plasmid location of sed can influence the mode of regulation of SED expression. It could be concluded that DNA amplification methods demonstrate the presence of SED expression. It could be concluded that DNA amplification methods demonstrate the presence of SEs in samples.

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References