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Development of a multiplex-PCR for direct detection of the genes for enterotoxin B and C, and toxic shock syndrome toxin-1 in Staphylococcus aureus isolates

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As well as conventional methods such as immunodiffusion, ELISA, or agglutination for the detection of toxin production in Staphylococcus aureus, amplification techniques like PCR allow a very sensitive and specific identification of the genes responsible for enterotoxin B and C, and TSST-1 production. These toxins might be a cause of the toxic shock syndrome (TSS). For that reason an easy and quick test system for determining the toxin production pattern of S. aureus isolates is desirable so that strains suspected to be toxin producers may be identified much faster and easier. In the present investigation, a new multiplex-PCR method was used that allowed single bacterial colonies grown on agar plates to be used directly in the PCR assay without preceding preparation. This procedure generated information concerning the presence of seb, sec-1 and tst genes within 4 h in a single test. To analyse the sensitivity and the specificity of this procedure, 100 methicillin-resistant S. aureus (MRSA), 50 coagulase-negative staphylococci and 50 other eubacterial isolates were tested initially with sets of single primer pairs followed by a combined multiplex-PCR. Results of this amplification technique were compared to a conventional and widely used method for toxin detection, reversed passive latex agglutination (RPLA). With the RPLA assay results as the basis, sensitivity and specificity of the seb and tst primer sets were 100%, whereas sensitivity and specificity of the sec-1 primer set were 100% and 82%, respectively. With the sec-1 primer set, two isolates were identified as carrying the corresponding toxin gene although the RPLA test did not show any detectable toxin. The multiplex-PCR rapidly generated reliable information concerning the toxin-producing capacity of staphylococcal strains and could be easily integrated into a multiplex procedure described previously. The latter enabled the identification of specific PCR products for eubacteria and staphylococci as well as the detection of the coa and mecA genes.

Introduction

Clinical isolates of Staphylococcus aureus are capable of producing a broad spectrum of extracellular toxins and virulence factors that contribute to their pathogenicity. Infections with S. aureus can cause the often fatal toxic shock syndrome (TSS). TSS can be caused by a number of toxins that act as super-antigens. These are proteins that strongly activate T lymphocytes and they possess two binding sites; one is the variable site for the antigen receptor of T lymphocytes, and the other is for the binding of MHC class II molecules. The activation of T lymphocytes causes the release of mediators (cytokines) that are responsible for the shock symptoms. First described in 1978, in relation to severe infections with S. aureus in children, the major symptoms are high fever, generalised exanthema, hypotension, multi-organ damage and desquamation of the skin. Between 1980 and 1986, 2960 cases of TSS in the USA were reported to the Centers for Disease Prevention and Control (CDC), of which almost 90% were associated with menses. The number
of cases of menstrual TSS has since decreased significantly, whereas the number of non-menstrual cases has remained relatively constant. Most *S. aureus* isolates from patients with TSS produce toxic shock syndrome toxin-1 (TSST-1). TSST-1 is a classical super-antigen that specifically activates T cells (Vβ-specific activation) [1–11].

TSS can occur during any extensive infection with *S. aureus* if sufficient amounts of toxins are produced and the patient does not possess protective levels of antibody. These toxins are active in the range of pg/ml. *S. aureus* produces large amounts, i.e., many micrograms, hence a local infection can introduce sufficiently high concentrations of toxin into the entire body. In addition to TSST-1, enterotoxins B and C can cause TSS. The genes for these three toxins are located on the bacterial chromosome. It is not known why enterotoxins A, D and E do not cause TSS. Among these five major enterotoxin serotypes (A–E), group C can be further distinguished into epitope types C1–3 [1–11]. More than 40% of all clinical isolates of *S. aureus* produce one or more super-antigens. On the other hand, strains have been isolated from patients with TSS that do not produce TSST-1 or enterotoxins B or C. This implies that there are other bacterial products that can cause TSS [11–18]. It is important to know whether an *S. aureus* isolate produces one or more of the toxins TSST-1, enterotoxin B, or enterotoxin C because it may help in the assessment of whether toxin-related symptoms are possible; it will not help to judge whether a strain may cause infection or just colonise a patient.

Conventional methods for the detection of enterotoxins and TSST-1 in culture media supernate are immuno-diffusion, agglutination and ELISA. Often, these methods do not allow the detection of very low concentrations of toxins and potential toxin-producing strains might not be discovered. Furthermore, the production of enterotoxins B and C and TSST-1 is influenced by the culture conditions (medium, pH, temperature, etc.) [19, 20]. Measured rates of toxin production always refer to particular culture conditions that might not represent conditions present in vivo. Hence the direct detection of genes for enterotoxin B (seb), C (sec) and TSST-1 (tst) is a clear indication of the toxin-producing capacity of a strain.

This report describes a multiplex-PCR technique that uses single colonies of *S. aureus* transferred directly from agar plates into the PCR assay for the parallel detection of seb, sec-1 and tst genes. Within 4 h, this test determines the genetic potential of the isolate for toxin production, while other available tests usually require at least 48 h to generate a result. Two aims of the present study were the evaluation of this multiplex-PCR for the parallel identification of seb, sec-1 and tst genes, and the comparison of multiplex-PCR with the commercially available and widely used agglutination assay. Previously, a multiplex-PCR was described [21] that allowed the detection of specific PCR products for eubacteria, staphylococci, the coa gene and the mecA gene. This method enabled single colonies taken directly from agar plates to be used in the PCR assay and generated a molecular dendrogram within 4 h that facilitated strain characterisation with specific information concerning taxonomy, pathogenicity and methicillin-resistance of staphylococci. A third aim of the present investigation was to combine both multiplex-PCRs into a single PCR assay to allow a rapid identification of staphylococcal strains and to determine their toxin-producing potential.

**Materials and methods**

**Strains**

One hundred MRSA isolates from seven countries (kindly provided by Hoffmann LaRoche, Basel, Switzerland) were analysed for their production of enterotoxins B and C and TSST-1. A further 50 coagulase-negative isolates and 50 other eubacterial isolates from clinical samples examined at the Düsseldorf Medical Institute for Medical Microbiology and Virology were analysed to determine the sensitivity and specificity of the technique. All the isolates (Table 1) have been described previously [21].

**Identification of S. aureus isolates.** The following criteria were used for the identification of *S. aureus* isolates: production of catalase; tube coagulase positivity (Bacto Coagulase Plasma EDTA-Test®, Difco, Augsburg, Germany); nuclease positivity (DNase agar, Oxoid, Wesel, Germany); anaerobic mannitol metabolism; identification in a commercial biochemical identification system (API-Staph®, bioMerieux, Nürtingen, Germany). Cultures of coagulase-positive, methicillin-susceptible and -resistant strains of *S. aureus* (ATCC nos. 12600, 13565, 19095, 25923, 29213, 33591, 33592 and 33593) and a coagulase-negative, methicillin-resistant strain of *S. epidermidis* (ATCC 27626) were used as controls.

**Susceptibility testing.** Resistance to oxacillin was determined by a disk diffusion method (DIN 58940) [22] (5-μg oxacillin disks, Becton Dickinson) on Mueller-Hinton agar supplemented with NaCl 2% and incubated for 48 h at 30°C. Resistance was defined as a zone of growth inhibition of ≤22 mm diameter.

**Enterotoxin test**

A commercially standardised test kit was used for the detection of staphylococcal enterotoxins B and C (SET-RPLA-Test® (Oxoid cat. no. TD 900), Unipath, Wesel, Germany) [23]. This test enabled the detection of enterotoxins in the culture medium of *S. aureus*. The method was based on reverse passive latex agglutination (RPLA).
Preparation of the culture media supernates. Four or five colonies of *S. aureus* were inoculated under shaking for 24 h at 37°C into 6 ml of casein peptone/soybean peptone medium and incubated at 37°C for 24 h with shaking. The cultures were centrifuged for 20 min at 4000 rpm at 4°C and 25 μl of the supernates were transferred into microtitre plates. The following steps were performed according to the manufacturer's instructions. The detection limit for enterotoxins given by the manufacturer is 0.5 ng/ml of culture media supernate. Lower concentrations give negative results.

Toxic shock syndrome toxin-1 test

A commercially standardised test kit was used for the detection of the staphylococcal TSST-1 in the culture medium (TST-RPLA-Test® (Oxoid cat. no. TD 940), Unipath) [23]. This method was also based on RPLA. The method and evaluation for this test were identical to the SET-RPLA-Test® (see above). The detection limit for TSST-1 given by the manufacturer is 2 ng/ml of culture media supernate. Lower concentrations give negative results.

Multiplex-PCR for the detection of genes *seb*, *sec-1* and *tst*

Selection of primers. Primers for this reaction were synthesised (Pharmacia Biotech) based on the published gene sequences for *seb* [24], *sec-1* [25] and *tst* [26]. When selecting the primers care was taken that no homologous regions between the individual genes could serve as primer targets. For enterotoxin C, three epitope differences were described [18]. Accordingly, primers were chosen to cover not only *sec-1*, but also *sec-2* and *sec-3*, all three having a homology of 98%. The following primer pairs were first tested individually before they were combined in the multiplex PCR assay.

*Seb* gene PCR for the detection of the enterotoxin B gene. 5’-Primer: 246-[5’]-GTATAAGAGATTATTTATTTCACTAG-[3’]-271 (26-mer); 3’-primer: 451-[5’]-TATATTAAGTCAGTAGAATTTG-[3’]-476 (26-mer). The amplification product has a size of 231 nucleotides.

*sec-1* gene PCR for the detection of the enterotoxin C-1 gene. 5’-Primer: 561-[5’]-CCACCTTTGATAAGGGGACTTAC-[3’]-583 (23-mer); 3’-primer: 808-[5’]-GATTGTCAAAATGTTAGCCTGG-[3’]-830 (23-mer). The amplification product has a size of 231 nucleotides.

*tst* gene PCR for the detection of the TSST-1 gene. 5’-Primer: 51-[5’]-AAGCCCTTGTGCTTCGGAC-[3’]-71 (21-mer); 3’-primer: 279-[5’]-AGCAGGGCTATAAAATAGGACTC-[3’]-300 (22-mer). The amplification product has a size of 250 nucleotides.

PCR protocol. Approximately one-tenth of single bacterial colonies grown for 24 h on Mueller-Hinton agar supplemented with sheep blood 5% were picked with a plastic pipette tip directly from the agar plate and gently mixed in the PCR amplification reaction mixture. The final volume of the PCR was 50 μl. Conditions for each PCR were as follows: 10 mmol Tris/HCl (pH 8.3), 50 mmol KCl, 2.5 mmol MgCl2, 100 μmol dNTPs and 0.4 μmol of each primer. The PCR reaction was performed in a GeneAmp PCR System 9200® (Perkin-Elmer); 3 U AmpliTaq-DNA Polymerase® were added to each reaction set-up after 5 min of the initial 10-min denaturation (94°C) step (hot start). This was followed by 25 amplification cycles of 94°C for 20 s, annealing at 55°C for 20 s, extension at 72°C for 50 s. The reaction was terminated by a 5-min final extension at 72°C.

*Agarose gel electrophoresis*. PCR reaction mix (20 μl) and 3 μl of sample buffer (glycerol 30%, BPB 0.1%) were loaded and separated in an agarose 3% w/v gel; 5 μl of a 1-kb ladder (BRL) was used as a mol.wt standard with every gel. Fragments were visualised after ethidium bromide staining under UV light.

Sensitivity testing. To test the sensitivity of the individual primer pairs, all *S. aureus* isolates were first tested with each of the primer pairs alone before testing with all primer pairs together within the multiplex-PCR. This was to show if all PCR products that were expected to be amplified because of the positive results in the RPLA-assay were readily formed both in the individual reaction and in the multiplex-PCR reaction.

Specificity testing. To test the specificity of the primer pairs, 50 coagulase-negative staphylococci and 50 other gram-positive and gram-negative clinical isolates were analysed in addition to the 100 MRSA strains.

Reproducibility. The reproducibility of the results was demonstrated by intra- and inter-assay analyses of three MRSA isolates, individually with each primer pair and with the primer pairs combined in the multiplex-PCR. During intra-assay analyses, three MRSA isolates were used 10 times after each other in the PCR. During the inter-assay analyses the same three isolates were tested on 10 subsequent days. Enterotoxin B, TSST-1 or enterotoxin C and TSST-1 were found in the supernates of the growth media of these three MRSA isolates.

Integration of both multiplex-PCR assays

As described previously [21] the multiplex-PCR was performed for the detection of specific PCR products for (1) eubacteria (*universal* 16s rRNA primer), (2) staphylococci (staphylococci-specific 16s rRNA-primer), (3) the coa gene (coa gene primer) and (4) the mecA gene (mecA gene primer). The primers for the detection of the *seb*, *sec-1* and *tst* genes were chosen to generate amplification products with lengths (231nt,
270nt and 250nt) that would integrate into the described method [21] without the bands overlapping on the agarose gel. After analysing all 200 isolates with each primer pair individually, followed by the first multiplex-PCR (to detect the toxin genes), all seven primer pairs were used together in the combined multiplex-PCR. Sensitivity, specificity and reproducibility of this combined method were evaluated as described above.

**Results**

**RPLA test**

With the RPLA test, 24 of the 100 MRSA strains produced enterotoxin B or C, or TSST-1 or combinations of these toxins. Enterotoxin B was detected in seven strains, enterotoxin C in nine strains, and TSST-1 in 17 strains in the culture media supernates. Among the 17 strains with TSST-1 in their culture supernates, nine strains also had enterotoxin C. No isolates were found that had both enterotoxins B and C, or enterotoxin B and TSST-1 in the supernate.

**Evaluation of the PCR systems**

First, the individual primer pairs for the amplification of regions in the seb, sec-1 and tst genes were tested individually with respect to their sensitivity and specificity with 100 MRSA, 50 coagulase-negative staphylococci and 50 other eubacterial isolates (Table 1). The results were identical with the combined approach in the multiplex-PCR. Also, the integrated multiplex-PCR with seven primer pairs produced identical results.

**Sensitivity.** The seb gene-specific PCR product was found in the seven strains that showed enterotoxin B in the RPLA assay. The sec-1 specific PCR product was found in 11 strains whereas the RPLA assay showed enterotoxin C in only nine strains. The tst gene-specific PCR product was found only in those 17 strains that showed the presence of TSST-1 in the RPLA assay.

**Specificity.** No seb-, sec-1 or tst-specific PCR products were obtained with any of the coagulase-negative staphylococci or other 50 eubacterial isolates. With RPLA assay results as the basis, sensitivity and specificity of the seb and tst primer sets were 100%, whereas sensitivity and specificity of the sec-1 primer set were 100% and 82%, respectively. Surprisingly, with the sec-1 primer set, two isolates were identified as carrying the corresponding toxin gene although the RPLA test did not detect any toxin in the culture supernates of these two isolates. The findings were confirmed by repeating both the PCR and the RPLA assay three times with these two isolates. The PCR product resulting from the use of the sec-1 primer set was sequenced to exclude false positive results in these two isolates. The sequence was identical with the published sequence of the sec-1 gene [18, 25].

**Reproducibility.** The PCR-based detection of toxin genes was fully reproducible. The reproducibility of intra- and inter-assay analyses was excellent. The specific PCR products of the three MRSA strains tested repeatedly were always detected when tested in the individual PCR systems, in the combined multiplex-PCR for toxin gene detection and in the integrated multiplex-PCR with seven primer pairs. Non-specific PCR products were not observed.

The figure shows some of the results of the PCR experiments. In lanes 2–4 the results obtained with separate primer pairs for seb, tst and sec-1 are shown for the isolates where enterotoxin B, TSST-1 or enterotoxin C were detected in the RPLA assay. Lanes 5–8 also show the results with the primer set for the detection of a staphylococci-specific PCR product (staphylococci-specific 16S rRNA primer set). If the toxin-PCR is not used as a multiplex-PCR with seven sets of primers, an internal amplification control

<table>
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<tr>
<th>Species</th>
<th>Number examined</th>
<th>Methicillin resistant</th>
<th>Methicillin susceptible</th>
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<tr>
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<td>10</td>
<td>Düsseldorf</td>
</tr>
<tr>
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<td>11</td>
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<tr>
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<td>2</td>
<td>2</td>
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<td>Proteus spp.</td>
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<td>Salmonella spp.</td>
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MULTIPLEx-PCR FOR DETECTION OF GENES seb, sec-1 AND tst

Fig. 1. Agarose gel electrophoresis of multiplex-PCR for the detection of seb, tst and sec-1 genes from S. aureus: Lane 1, mol. wt standard (1-kb ladder); 2, specific PCR product for the seb gene; 3, specific PCR product of the tst gene; 4, specific PCR product for the sec-1 gene; 5, staphylococci-specific PCR product; 6, staphylococci-specific PCR product with the tst gene-specific PCR product; 7, staphylococci-specific PCR product with the seb gene-specific PCR product; 8, staphylococci-specific PCR product with the tst and sec-1 gene-specific PCR product; 9, specific PCR products for eubacteria, sec-1 gene, tst gene, mecA gene, coa gene and for staphylococci (from largest to smallest PCR product); 10, E. coli control; 11, distilled water control; 12, mol. wt standard (1-kb ladder).

is necessary (e.g., staphylococci-specific 16S rRNA primer set). Lane 9 shows the result of an integrated multiplex-PCR reaction with seven primer sets on an MRSA strain that produced TSST-1 and enterotoxin C according to the RPLA assay. As expected, it shows all the specific PCR products for eubacteria, for staphylococci and for the genes for coa, mecA, tst and sec-1. Lane 10 shows a multiplex-PCR with primers seb, sec-1 and tst on an Escherichia coli strain (no PCR products), and lane 11 is a control with distilled water for the integrated multiplex-PCR reaction with seven primer sets.

Discussion

Several studies have shown that 25–40% of S. aureus isolates produce at least one of the three super-antigens TSST-1, enterotoxin B or enterotoxin C [11–18]. Under particular circumstances, TSS could result from at least one out of four infections with S. aureus. As mentioned above, it is a combination of several factors that can cause TSS.

Classical methods for the detection of enterotoxins and TSST-1 in culture supernates are immunodiffusion, agglutination and ELISA. These methods are time consuming and do not always detect toxin-producing strains because toxin production partly depends on culture conditions that do not represent conditions in vivo [19,20]. Furthermore, immunological methods can produce false-positive results through cross-reacting antigens. These disadvantages can be circumvented by direct detection of the genes seb, sec and tst responsible for toxin production. If the toxin-producing gene is detected, the possibility of toxin production has to be considered. The detection of these toxin genes does not necessarily indicate that the strain is producing the toxin. The gene might be present in an inactivated form or other factors may be missing that are required for gene expression.

The results of the toxin gene detection PCR and the RPLA assay compared well with each other. The seb- and tst-specific PCR products were detected correctly in all enterotoxin B- and TSST-1-producing isolates. Also, the sec-1-specific PCR product was detected in all enterotoxin C-producing isolates, but it was also found in two strains that did not produce any detectable amounts of enterotoxin C in the RPLA assay. In such cases, either the in-vitro toxin production was below the detection limit of the RPLA or the sec-1 gene was inactivated (e.g., by a point mutation with insertion of a stop codon), or other factors for in-vitro toxin production were absent. For practical clinical considerations, toxin gene-positive
staphylococci should be considered to be toxin producers because in-vivo toxin production cannot be excluded. Sensitivity and specificity of the primer pairs was excellent, as well as reproducibility of the multiplex-PCR results. It should be emphasised that it is important for clinical investigation to have a rapid, reliable method for the determination of the presence of seb, sec and tst genes.

The applicability of PCR and hybridisation assays for the detection of toxin genes has been shown before [27–29]. Also, as in the present study, results have compared well with the RPLA assay. The following are the advantages of the PCR method for the detection of the seb, sec1 and tst genes. (1) Use of single colonies from the agar plate without further subculturing; no DNA extraction, or enzyme incubation required, but cells can be directly introduced into the PCR assay. (2) Simultaneous detection of the three genes (seb, sec1 and tst) with the multiplex-PCR; parallel use of several PCR reactions in one set-up facilitates the routine application in the clinical laboratory. (3) Combining individual PCR reactions into an integrated multiplex-PCR with seven primer pairs to identify enterotoxins B and C and TSST-1, identify S. aureus among other staphylococci by the detection of the coa gene and test for methicillin resistance by the detection of the mecA gene. (4) A fast method through its simple and reliable protocol; time requirements are typically in the range of 4 h only. The method represents a novel and alternative approach for the detection of toxin production in S. aureus. The possibility to perform it within a multiplex-PCR conveniently integrates this method into the work-flow of a routine microbiology laboratory.

References