Use of Multiplex PCR To Detect Classical and Newly Described Pyrogenic Toxin Genes in Staphylococcal Isolates

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Staphylococcus aureus may contain one or more genes that encode a variety of immunomodulatory pyrogenic toxins (PTs), including the staphylococcal enterotoxins and toxic shock syndrome toxin (TSST). The PTs interact with several cellular targets to produce disease, such as food poisoning and toxic shock syndrome. At present, none serologically distinct enterotoxins and one immunoreactive form of TSST have been identified and characterized. As isolates of S. aureus are further assessed, it is anticipated that this number will increase. To facilitate screening, a multiplex PCR was designed to simultaneously determine which of these 10 currently known PT genes an individual S. aureus isolate possesses. We show here, using S. aureus isolates with characterized PT phenotypes, that this novel PCR technique reliably detects each of the known PTs in a single reaction.

Staphylococcus aureus is a common pathogen that colonizes and produces disease in a variety of hosts. The ability of this bacterium to successfully persist within this range of hosts is largely due to the expression of a battery of virulence factors which promote adhesion, acquisition of nutrients, and evasion of host immunologic responses (18). Among these is the pyrogenic exotoxin (PT) family, which is comprised of several structurally and biologically related proteins expressed by both S. aureus and Streptococcus pyogenes (22).

PTs, which include toxic shock syndrome toxin (TSST) and the staphylococcal enterotoxins (SEs), are secreted proteins that interact with antigen-presenting cells and T lymphocytes to induce cellular proliferation (12) and high-level cytokine expression (9). This activity does not involve the endocytic processing required for typical antigen presentation but instead occurs by concurrent association with major histocompatibility complex class II molecules of the antigen-presenting cells and the Vβ domain of the lymphocyte T-cell receptor (13). This interaction activates a much greater percentage of the host T-cell repertoire than that induced by antigens presented in the traditional manner (15), explaining the massive cytokine expression and subsequent immunomodulation brought about by these toxins. Proteins which have the capacity to interact with the host immunological system in this manner have been termed superantigens (SAgs), and the PTs are prototypic examples of bacterial SAgs (10).

The SEs and TSST are the causative agents of toxic shock syndrome (6). Additionally, unlike the other members of the PT family, the SEs have the unique ability to induce staphylococcal food poisoning, a common form of gastroenteritis (10). Presently, nine major antigenic types of SEs have been reported (SEA, SEB, SEC, SEĐ, SEE, SEG, SEH, SEI, and SEJ), while only one serotype of TSST (comprising of TSST-1 and TSSTovine) has been described (1, 4, 5, 8, 19, 20, 24, 25). The SEc serotype is heterogeneous and contains several antigenic and sequence molecular variants, designated SEC1, SEC2, SEC3, SECbovine, and SECovine. These have been classified on the basis of minor antigenic differences and the animal host with which they are associated (17). Because of the significance of these toxins for public health and food safety, an efficient means for screening is needed. Also, since several of these toxins have been discovered in very recent years, there is reason to believe that as research on pathogenic S. aureus isolates continues, additional SAgs will be described. Identification of novel toxins will require an efficient means to screen isolates for previously described staphylococcal PT genes. Toxigenic isolates that do not harbor genes for currently recognized toxins are likely to express novel SAgs. Therefore, we developed a multiplex PCR procedure which will rapidly and simultaneously assess whether staphylococcal isolates harbor sea, seb, sec, sed, see, seg, seh, sei, and sej, encoding the SEs, and tst, which encodes TSST.

Bacterial strains and DNA isolation. Developing and testing of the multiplex procedure was accomplished with DNA from the bacterial strains listed in Table 1. Collectively, these isolates contain all of the previously reported PT genes. Staphylococcal genomic DNA was obtained from lysostaphin-treated cells by a previously described process (7). The DNA was extracted with phenol and chloroform and was ethanol precipitated by standard methods (21). The DNA was recovered by centrifugation, vacuum dried, resuspended in 200 μl of pyrogen-free H2O, quantified spectrophotometrically at 260 and 280 nm, and diluted to a final concentration of 10 ng/μl.

PCR primer design and amplification of bacterial DNA. Nucleotide sequences for each of the PT genes were obtained from GenBank by using their specific accession numbers (Table 2). The sequences were compared and evaluated by using Genetics Computer Group (Madison, Wis.) computer software to identify nucleotide sequences unique to each gene. With the exception of the seb-sec primer set, which produces a 643-bp amplification product common to both seb and sec, all primer sets were designed to anneal to unique regions and generate amplification products that would allow identification of each PT gene based on the molecular weight of its PCR product (Table 2). To discriminate whether the 643-bp seb-sec amplification product is indicative of either seb or sec, a separate 5′ sec primer was designed that works in combination with the 3′ seb-sec primer to produce a 283-bp amplification product unique to sec, including the bovine variant (see below). Additionally, to ensure that toxin-negative samples were interpreted correctly and that a sufficient quantity of PCR template DNA

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was present, the samples were also tested by PCR with a primer set that anneals to the *S. aureus* 16S rRNA gene that generates a 228-bp amplicon during the amplification process (Table 2).

The multiplex PCR was performed in a 50-μl volume with the Gibco BRL *Taq* DNA polymerase system (Life Technologies, Inc., Rockville, Md.) containing the following: 1× *Taq* polymerase buffer, 4 mM MgCl₂, 300 nM concentrations of each of the primers listed in Table 2, 400 μM concentrations of deoxynucleoside triphosphates, 5 U of *Taq* polymerase, and 50 ng of staphylococcal DNA. Bulk solutions containing the *Taq* buffer, MgCl₂, deoxynucleoside triphosphates, and multiplex PCR primer mix at the appropriate concentrations were prepared for the desired number of reactions. Aliquots of the staphylococcal template DNA (50 ng in 5 μl) from each test strain were individually placed into 500-μl thin-walled PCR tubes. Afterwards, 40 μl of the bulk solution was added to each tube containing the template DNA and covered with 50 μl of mineral oil. These tubes were subsequently incubated for 10 min (95°C), during which (after the initial 3 min) 5 U of *Taq* polymerase (in 5 μl [total volume] of 1× *Taq* buffer) was added to each reaction. Following this “hot-start” procedure, DNA was amplified in an Amplitron II thermocycler (Barnstead Thermolyne Co., Dubuque, Iowa) by 15 cycles of 95°C for 1 min, 68°C for 45 s, and 72°C for 1 min and 16 cycles of 95°C for 1 min, 64°C for 45 s, and 72°C for 1 min. The reaction was terminated with a 10-min incubation at 72°C.

PCR products were resolved by electrophoresis in 1.5% agarose (0.5× Tris boric acid, EDTA) gels at 100 V (constant voltage) and visualized on a transilluminator with a charged coupled device camera and the Molecular Analyst software (Bio-Rad, Hercules, Calif.). Product sizes were determined by using the 1 Kb Plus DNA molecular weight ladder (Life Technologies, Inc.).

**Genetic analysis of clinical strains using the multiplex PCR procedure.** Analyses using DNA obtained from the staphylo-

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**TABLE 1. *S. aureus* strains used in multiplex PCRs**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Toxin genotype determined by:</th>
<th>Reference</th>
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<tr>
<td></td>
<td>Previous work</td>
<td>Multiplex PCR</td>
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<tr>
<td>RN4220</td>
<td>Nontoxicogenic control</td>
<td>Nontoxicogenic</td>
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<tr>
<td>FR913</td>
<td>sea sec see tst</td>
<td>see sec see tst</td>
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<td>MNHOCH</td>
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<td>seb</td>
</tr>
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<td>sed</td>
<td>sed seg sei sej</td>
</tr>
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<tr>
<td>FR572</td>
<td>seg</td>
<td>seg sei</td>
</tr>
<tr>
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<td>seh</td>
<td>seh</td>
</tr>
<tr>
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<td>sei</td>
<td>seg sei</td>
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<tr>
<td>3169</td>
<td>tst sec</td>
<td>sec sec see tst</td>
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</tbody>
</table>

* FRI, Food Research Institute, University of Wisconsin—Madison.

**TABLE 2. Staphylococcal toxin-specific primers used for multiplex PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’ and 3’)</th>
<th>GenBank accession no.</th>
<th>Location</th>
<th>Size</th>
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<tr>
<td>sea</td>
<td>GCA GGG AAC AGC TTG AGG C</td>
<td>M18970</td>
<td>126–144</td>
<td>520</td>
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<td></td>
<td>GTT CTG TAG AAG TAT GAA ACA</td>
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<td>646–624</td>
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<td></td>
<td>AGC GAT CAT CAC ACA CG</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>seb-sec</td>
<td>ATG TAA TTT TGA TAT TCG CAG TG</td>
<td>M11118 (seb)</td>
<td>28–48</td>
<td>643</td>
</tr>
<tr>
<td></td>
<td>TGC AGG CAT CAT ATA CCA</td>
<td></td>
<td>690–670</td>
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</tr>
<tr>
<td>sec</td>
<td>CTT GTA TGT ATG GAG GAA TAA CAA</td>
<td>X05815</td>
<td>407–430</td>
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<tr>
<td></td>
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<tr>
<td>sed</td>
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<td>M28521</td>
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<td>384</td>
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<td>ATA TGA AGG TGC TCT GTG G</td>
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<tr>
<td>see</td>
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<td>M21319</td>
<td>446–468</td>
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<td>TCT TTT GCA CCT TAC CGC</td>
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<td>seg</td>
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<td>seh</td>
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<td>sei</td>
<td>CAA CTC GAA TTT TCA ACA GGT AC</td>
<td>AF064774</td>
<td>325–347</td>
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<tr>
<td>sej</td>
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<td>CTC GTA AAT TTT ACC ATC AAA GGT AC</td>
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<td>TGG ATC CGT CAT TCA TGT TTA A</td>
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<td>16S rRNA</td>
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<td>X68417</td>
<td>545–564</td>
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<td></td>
<td>CGC ACA TCA GTC GTC AG</td>
<td></td>
<td>773–758</td>
<td></td>
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</tbody>
</table>

* Primer sequences are given in the 5’—3’ direction. For each primer pair the sequence of the 5’ primer is provided first (above), followed by that of the 3’ primer (below).

* Staphylococcal PT gene identical to designation in first column unless otherwise noted in parentheses.

* Location of primer sequence within open reading frame using the nucleotide numbering indicated in GenBank.

* Predicted PCR product size generated by using the indicated gene-specific primer pair. For instance, the *sea* primer pair produces a 520-bp amplicon when the gene encoding SEA is present in the reaction mixture.
The multiplex PCR process described in this report reliably detects the genes for all staphylococcal PTs reported as of April 1999. Moreover, this technique was shown to be able to detect at least four different SE genes (producing five separate PCR products) in a single bacterial isolate. The fact that it can simultaneously detect all 10 genes in a pooled sample of DNA ensures that this procedure can both confirm the presence of PT genes previously associated with a particular strain and detect other currently known toxin genes within the isolate. For instance, in the present study *S. aureus* FRI472, previously described as a SED producer (1), consistently generated a 384-bp PCR product, confirming the presence of the *sed* gene within its genome. However, amplicons with molecular sizes of 143, 327, and 465 bp were also produced, indicating that FRI472 contains genes encoding SEJ, SEG, and SED, respectively, as well (Fig. 1). This observation is consistent with the recent report by Zhang et al. (25), who determined that the SEJ determinant is present on the same plasmid as the SED determinant. Similarly, we also showed that other isolates, such as *S. aureus* FR1572 and FR1445, carry toxin genes not previously associated with these isolates (Fig. 1).

This work has produced a system that expands the capabilities of the multiplex PCR procedures previously developed by several other investigators (2, 11, 14, 23). Most notably, the system described in this report reliably, rapidly, and simultaneously detects each of the 10 currently described staphylococcal toxin determinants, including the most recently described *seg* (19), *sei* (19), and *sej* (25) genes. Additionally, in a single reaction, the process generates amplicons that allow easy discrimination of the determinants an isolate carries, regardless of the number of PT genes carried by the isolate. These features allow this procedure to be applied in the clinical setting for epidemiological studies or to guide therapeutic strategies. This efficient method of screening isolates for PT genes could also facilitate the identification of additional genes encoding novel, yet-undescribed toxins.

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**REFERENCES**


