JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1999, p. 3411–3414 Vol. 37, No. 10
0095-1137/99/$04.00+0

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

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Received 21 April 1999/Returned for modification 8 June 1999/Accepted 14 July 1999

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, nine 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, SED, SEE, SEG, SEH, SEI, and SEJ), while only one serotype of TSST (comprised of TSST-1 and $TSST_{ovine}$) has been described (1, 4, 5, 8, 19, 20, 24, 25). The SEC serotype is heterogenous and contains several antigenic and sequence molecular variants, designated SEC1, SEC2, SEC3, SEC_{bovine}, and SEC_{ovine}. 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 H_2O, 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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TABLE 1. *S. aureus* strains used in multiplex PCRs

Strain[a]	Toxin genotype determined by:		Reference
	Previous work	Multiplex PCR	
RN4220	Nontoxigenic control	Nontoxigenic	16
FRI913	*sea sec see tst*	*sea sec see tst*	3, 17
MNHOCH	*seb*	*seb*	7
FRI472	*sed*	*sed seg sei sej*	16
FRI326	*see*	*see*	16
FRI572	*seg*	*seg sei*	19
FRI569	*seh*	*seh*	23
FRI445	*sei*	*seg sei*	19
3169	*tst* sec_{bovine}	sec_{bovine} *sed sej tst*	17

[a] FRI, Food Research Institute, University of Wisconsin—Madison.

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 $MgCl_2$, 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, $MgCl_2$, 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-

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

Gene	Primer sequence (5′ and 3′)[a]	GenBank accession no.[b]	Location[c]	Size[d]
sea	GCA GGG AAC AGC TTT AGG C GTT CTG TAG AAG TAT GAA ACA CG	M18970	126–144 646–624	520
seb-sec	ATG TAA TTT TGA TAT TCG CAG TG TGC AGG CAT CAT ATC ATA CCA	M11118 (*seb*)	28–48 690–670	643
sec	CTT GTA TGT ATG GAG GAA TAA CAA TGC AGG CAT CAT ATC ATA CCA	X05815	407–430 690–670	283
sed	GTG GTG AAA TAG ATA GGA CTG C ATA TGA AGG TGC TCT GTG G	M28521	368–389 752–734	384
see	TAC CAA TTA ACT TGT GGA TAG AC CTC TTT GCA CCT TAC CGC	M21319	446–468 616–599	170
seg	CGT CTC CAC CTG TTG AAG G CCA AGT GAT TGT CTA TTG TCG	AF064773	317–335 644–624	327
seh	CAA CTG CTG ATT TAG CTC AG GTC GAA TGA GTA ATC TCT AGG	U11702	245–264 603–583	360
sei	CAA CTC GAA TTT TCA ACA GGT AC CAG GCA GTC CAT CTC CTG	AF064774	325–347 790–773	465
sej	CAT CAG AAC TGT TGT TCC GCT AG CTG AAT TTT ACC ATC AAA GGT AC	AF053140	471–493 612–590	142
tst	GCT TGC GAC AAC TGC TAC AG TGG ATC CGT CAT TCA TTG TTA A	J02615	48–67 606–587	559
16S rRNA	GTA GGT GGC AAG CGT TAT CC CGC ACA TCA GC GTC AG	X68417	545–564 773–758	228

[a] 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).

[b] Staphylococcal PT gene identical to designation in first column unless otherwise noted in parentheses.

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

[d] 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.

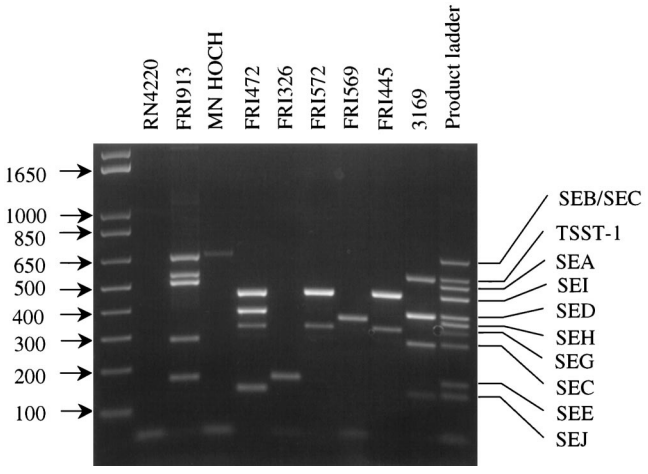

FIG. 1. Agarose gel electrophoresis of the multiplex PCR amplification products from analysis of bacterial test strains. Lanes contain amplification products of DNA isolated from strains designated at top. Product identification was facilitated by direct comparison to the 1 Kb Plus (Gibco BRL) molecular weight ladder (far left lane) and a PT gene amplification product ladder prepared by pooling reaction products generated in individual reactions (far right lane).

coccal isolates demonstrated that all primer pairs produced amplification products consistent with their predicted sizes. For example, amplification of DNA from FRI913, known to produce SEA, SEC, SEE and TSST-1 (3), generated bands indicative of *sea* (520 bp), *sec* (643 and 283 bp), *see* (170 bp), and *tst* (559 bp) (Fig. 1). Similarly, amplification of DNA obtained from *S. aureus* MN HOCH (7), FRI472 (16), FRI326 (16), FRI572 (19), FRI569 (24), and FRI445 (19) produced PCR products consistent with the toxin genes that had previously been reported for these strains (Table 1 and Fig. 1). As expected, while amplification of DNA obtained from the nontoxigenic strain RN4220 consistently produced the 16S rRNA gene amplicon (results not shown), it failed to produce any SE-related PCR product when subjected to the multiplex PCR analysis. Since a single isolate may contain multiple toxin genes, we assessed the number of genes which could be simultaneously detected with this process under these particular reaction conditions. Using DNA pooled from several isolates (FRI913, FRI472, and FRI569), we demonstrated that it was possible to simultaneously generate PCR products representative of all 10 PT genes, as well as the 16S rRNA gene, in a single reaction (results not shown).

By design, amplification of DNA obtained from strains producing SEC were expected to result in the production of the 643- and 283-bp amplification products indicative of *sec*, like that observed for FRI913 (Fig. 1). However, strains known to express SEC_{bovine} did not generate both amplicons when tested by this procedure. Amplification of DNA obtained from the SEC_{bovine}^{+} strain 3169 (Fig. 1) (17) resulted in the production of the 283-bp amplicon but did not produce the 643-bp *seb-sec* PCR product typically observed in analyses of SEB- or SEC-expressing isolates (MN HOCH and FRI913, respectively) (Fig. 1). This finding, which was also observed when DNA isolated from another known SEC_{bovine}-producing *S. aureus* isolate was tested (results not shown), suggests that the gene encoding SEC_{bovine} has unique attributes not shared by other *sec* genes (see below). The 5′ *seb-sec* primer was designed to anneal to these genes at a conserved sequence within the region encoding the signal peptides of SEB and SEC. Since amplification of DNA derived from SEC_{bovine} producers does not result in the production of the 643-bp amplicon, it is likely that the region of the gene encoding the SEC_{bovine} signal peptide is comprised of a nucleotide sequence dissimilar to that of the other *sec* variants. At present, the nucleotide sequence within this portion of the SEC_{bovine} gene is not available in GenBank and, therefore, cannot be directly compared to analogous regions of the other *sec* variants.

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 SEI, 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* FRI572 and FRI445, 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.

This work was supported by the funds provided by Public Health Service grant AI28401, the United Dairymen of Idaho, and the Idaho Agriculture Experiment Station (G.A.B.).

We thank Claudia Deobald for her assistance in the development of this assay. We also thank Patrick Schlievert (at the University of Minnesota), Amy Wong (at the Food Research Institute at the University of Wisconsin), and Merlin Bergdoll (also at the Food Research Institute) for kindly providing many of the strains used in this work.

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