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Detection of Genes for Enterotoxins, Exfoliative Toxins, and Toxic Shock Syndrome Toxin 1 in *Staphylococcus aureus* by the Polymerase Chain Reaction

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Eight pairs of synthetic oligonucleotide primers were used in a polymerase chain reaction (PCR) protocol to detect genes for staphylococcal enterotoxins A to E, exfoliative toxins A and B, and toxic shock syndrome toxin 1 in *Staphylococcus aureus* strains isolated from clinical specimens and contaminated foods. Primers were targeted to internal regions of the toxin genes, and amplification fragments were detected after the PCR by agarose gel electrophoresis. Unequivocal discrimination of toxin genes was obtained by the PCR by using nucleic acids extracted from 88 strains of *S. aureus* whose toxigenicity was established biologically and immunologically. In immunological assays, two strains of *S. aureus* produced equivocal results for production of enterotoxin C or toxic shock syndrome toxin 1, giving an overall concordance between phenotypic and genotypic identification of 97.7%. Primer specificity was established in the PCR by using nucleic acids from known toxin-producing bacterial pathogens and from nontoxigenic *S. aureus*. Strains of *Streptococcus* spp., including some producers of pyrogenic exotoxin A carrying the *speA* gene, were negative by the PCR designed to detect staphylococcal toxins. The detection limits were established for all the staphylococcal toxin genes within their respective PCR protocols. The identification of staphylococcal toxin genes in strains of *S. aureus* by the PCR offers a very specific, sensitive, relatively rapid, and inexpensive alternative to traditional immunological assays which depend on adequate gene expression for reliability and sensitivity.

Clinical isolates of *Staphylococcus aureus* can produce a spectrum of extracellular protein toxins and virulence factors which are thought to contribute to the pathogenicity of the organism. The staphylococcal enterotoxins are recognized agents of intoxication staphylococcal food poisoning syndrome and may be involved in other types of infections with sequelae of shock in humans and animals (3, 19). Serologically, five toxin groups have been recognized and designated staphylococcal enterotoxin A (SEA), SEB, SEC, SED, and SEE. Minor epitope differences in the SEC group have resulted in a further subdivision into SEC1, SEC2, and SEC3 (3). SEA, SED, and SEE share immunological determinants, as do SEB and SEC1 and streptococcal pyrogenic exotoxin A (SPEA) (7, 14, 19).

The genetics of *S. aureus* enterotoxin production have been well studied (1, 2, 4, 7, 9, 13, 15), and genes coding for these toxins have been localized on the chromosome for SEB and SEC, on bacteriophage vectors for SEA, and on plasmids for SED. Although SEE and SEA are the most closely related enterotoxins (9), SEE genes have not been shown to be phage associated, and hybridization data suggest that genes for SEE may be linked to a defective converting phage carrying SEA-like coding sequences.

Clinically, toxic shock syndrome is closely associated with strains of *S. aureus* carrying the *tst* gene encoding toxic shock syndrome toxin 1 (TSST-1) (27). The *tst* gene is chromosomal, and the toxin is symptomatically related to the staphylococcal enterotoxin group of toxins (5, 13). However, no immunological identity and little amino acid homology between TSST-1 and the staphylococcal enterotoxins or pyrogenic exotoxins exist (5, 19).

Some strains of *S. aureus* producing one or both of two immunologically distinct exfoliative toxins, exfoliative toxin A (ETA) or ETB (13, 17, 19), have been associated with a series of impetiginous staphylococcal diseases referred to as staphylococcal scalded skin syndrome. Although ETA and ETB have identical biological activity and a degree of genetic similarity (19), the gene coding for ETA is chromosomal whereas the gene coding for ETB is plasmid linked (17).

In the present study, we report the development of polymerase chain reaction (PCR) procedures which will rapidly and specifically detect genes for staphylococcal enterotoxins A to E, TSST-1, and exfoliative toxins A and B in strains of toxigenic *S. aureus* associated with human disease. These PCR protocols were performed by using template nucleic acids (NA) extracted from a collection of 88 strains of *S. aureus* isolated from both human and nonhuman sources. PCR results were compared with the results of biological assays for SEA, SEB, SEC, SED, SEE, TSST-1, ETA, and ETB.

MATERIALS AND METHODS

Bacterial strains and culture media. A phenotypic list of the *S. aureus* strains used in this study is presented in Table 1. Clinical isolates, isolates from foods, and reference strains were stored on suitable maintenance media and collected by the National Laboratory for Bacteriology, Laboratory Centre for Disease Control. Reference strains had been previously defined in terms of toxigenicity with respect to enterotoxins, exfoliative toxins, and toxic shock syndrome toxin. The following strains were included in this study: ATCC 13565 (SEA), ATCC 14458 (SEB), ATCC 19095 (SEC), ATCC 23235 (SED), and ATCC 27664 (SEE). Bacterial

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TABLE 1. Summary of *S. aureus* toxin phenotypes and PCR amplification results

Toxin phenotype	No. of strains	No. positive by:		PCR amplification product size(s) (bp)
		PCR	Immunoassay	
SEA	5	5	5	120
SEB	6	6	6	478
SEC	7	7	7	257
SED	4	4	4	317
SEE	2	2	2	170
SEA + SEB	3	3	3	120, 478
SEA + SED	2	2	2	120, 317
SEC + SED	4	4	4	257, 317
TSST-1	4	4	4	350
SEA + TSST-1	14	14	14	120, 350
SEB + TSST-1[a]	3	3	2	478, 350
SEC + TSST-1[b]	7	7	6	257, 350
ETA[c]	7	7	7	119
ETB	2	2	2	200
ETA+ ETB	1	1	1	119, 200
Negative[d]	17	0	0	None

[a] For one strain, the result of the immunoassay was equivocal but the PCR clearly identified TSST-1.
[b] For one strain, the result of the immunoassay was equivocal but the PCR clearly identified SEC.
[c] Three of these strains were previously identified as ETA- and ETB-producing strains.
[d] Four strains previously identified as exfoliative toxin producers were negative by both the PCR and immunodiffusion tests.

TABLE 2. Base sequences, locations within the genes, and predicted sizes of amplified products for the staphylococcal toxin-specific oligonucleotide primers

Gene[a]	Primer[b]	Oligonucleotide sequence (5′–3′)	Location within gene[c]	Size of amplified product (bp)
sea	SEA-1	ttggaaacggttaaaacgaa	490–509	
	SEA-2	gaaccttcccatcaaaaaca	591–610	120
seb	SEB-1	tcgcatcaaactgacaaacg	634–653	
	SEB-2	gcaggtactctataagtgcc	1091–1110	478
sec-1	SEC-1	gacataaaagctaggaattt	676–695	
	SEC-2	aaatcggattaacattatcc	913–932	257
sed	SED-1	ctagtttggtaatatctcct	354–373	
	SED-2	taatgctatatcttataggg	652–671	317
see	SEE-1	tagataaagttaaaacaagc	491–510	
	SEE-2	taacttaccgtggacccttc	640–659	170
tst	TSST-1	atggcagcatcagcttgata	251–270	
	TSST-2	tttccaataaccacccgttt	581–600	350
eta	ETA-1	ctagtgcatttgttattcaa	374–393	
	ETA-2	tgcattgacaccatagtact	473–492	119
etb	ETB-1	acggctatatacattcaatt	51–70	
	ETB-2	tccatcgataatatacctaa	231–250	200

[a] Sequences and locations derived from the published nucleotide sequences for SEA (4), SEB (15), SEC (7), SED (2), SEE (9), TSST-1 (5), and ETA and ETB (17).
[b] All primers whose designations end in "-2" are complementary sequences to the specified gene locations.
[c] Given in nucleotide numbers.

cultures were grown in brain heart infusion broth prior to extraction of total NA.

Determination of staphylococcal enterotoxins, exfoliative toxins, and toxic shock syndrome toxin. Culture filtrates of *S. aureus* were tested for the presence of enterotoxins A, B, C, and D by using a semiquantitative reversed passive latex agglutination (RPLA) toxin detection kit (SET-RPLA; Oxoid Ltd., Basingstoke, Hampshire, England). The presence of enterotoxin E was determined by E. Todd, Bureau of Microbial Hazards, Health and Welfare Canada, by using a modified Ouchterlony microslide test (25). RPLA tests were also used for the identification of staphylococcal toxic shock syndrome toxin (TST-RPLA; Oxoid Ltd.). Exfoliative toxins A and B were detected by immunodiffusion tests (10) by using toxin-specific antisera supplied by J. P. Arbuthnott, Department of Microbiology, University Hospital, Queen's Medical Centre, Nottingham, England. Some of the *S. aureus* strains producing exfoliative toxins A and B were provided by M. E. Melish, Kapiolani-Children's Medical Center, University of Hawaii at Manoa, Honolulu, who had previously confirmed their toxigenicity by the formation of a Nikolsky sign in the neonatal mouse bioassay (10).

NA isolation. Total NA were isolated from 5 ml of an 18-h broth culture from all the bacterial strains listed in Table 1. Cells were pelleted from the cultures by centrifugation at 1,000 × *g* for 10 min (IEC Centra 7R centrifuge), resuspended in phosphate-buffered saline with 100 μg of lysostaphin (Sigma) per ml, and incubated at 37°C for 0.5 h or until viscous. NA from all preparations were subsequently extracted with phenol-chloroform and precipitated with ethanol (18). NA samples were dissolved in TE buffer (10 mM Tris chloride–1 mM EDTA [pH 8.0]) and adjusted to a final concentration of 2 μg/ml with TE buffer according to A_{260} values.

PCR. Oligonucleotide primers were designed by computerized sequence analysis (11) and obtained from the Oligonucleotide Synthesis Laboratory, Queen's University, Kingston, Ontario, Canada. The PCR was performed in a 50-μl reaction mixture as previously described (23) by using 10 ng of NA with the following amplification cycles: denaturation for 2 min at 94°C, annealing of primers for 2 min at 55°C, and primer extension for 1 min at 72°C with autoextension. All PCR protocols were performed for individual toxin genes by using only one primer pair for each tube. To test the sensitivity of the PCR procedure in detecting the staphylococcal toxin genes, NA from strains of *S. aureus* carrying the specific toxin genes were adjusted to a concentration of 200 μg/ml, and serial 10-fold dilutions were made in TE buffer before the NA were used as templates in the PCR.

RESULTS

Specificity and sensitivity of the staphylococcal oligonucleotide primers targeting toxin genes. Eight pairs of synthetic toxin-specific oligonucleotide primers directed at specific nucleotide sequences internal to the toxin genes (Table 2) were used in the PCR procedure. The primers were selected by computerized analysis by using published sequences for the *entA* (4), *entB* (15), *entC*$_1$ (7), *entD* (2), *entE* (9), *tst* (5), and *eta* and *etb* (17) genes. The new staphylococcal gene nomenclature is used here to refer to genes coding for SEA (*sea*), SEB (*seb*), SEC (*sec*), SED (*sed*), and SEE (*see*). Figure 1 shows the presence and occurrence of the amplified products (amplicons) after agarose gel electrophoresis when NA extracted from representative toxigenic *S. aureus* were used as templates in the PCR. The sizes of the amplicons were identical to those predicted from the design of the

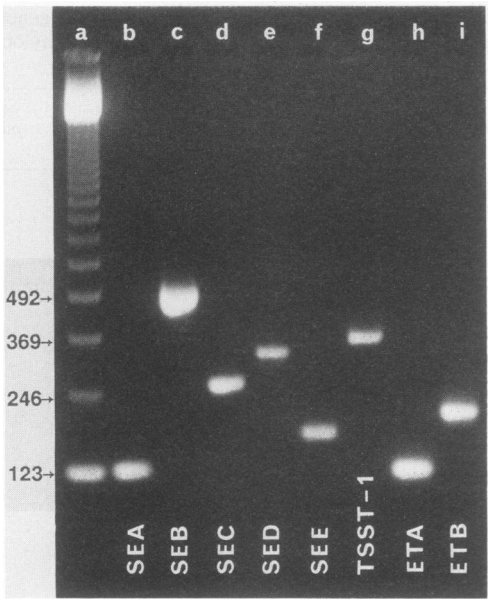

FIG. 1. Agarose gel electrophoresis patterns showing typical amplification fragments in the PCR for the staphylococcal toxin genes. Lanes: a, 123-bp ladder (Bethesda Research Laboratories); b, SEA; c, SEB; d, SEC; e, SED; f, SEE; g, TSST-1; h, ETA; i, ETB.

primers (Tables 1 and 2). The amplicons were not subjected to further identification procedures since all of the target nucleotide sequences were internal to the coding region for the toxin genes, the observed sizes of the amplification fragments were identical to the predicted theoretical sizes, and the NA were extracted from pure cultures of well characterized strains of *S. aureus*. There is, however, ample opportunity for restriction analysis because of the presence of various restriction endonuclease sites within each amplicon. No toxin-specific amplification fragments were observed in the PCR by using template NA from nontoxigenic *S. aureus*. The sensitivity limits of this application of the PCR for detecting staphylococcal toxin genes in *S. aureus* NA were 100 pg for *tst*, 10 pg for *sea*, *sec-1*, *sed*, *see*, *eta*, and *etb*, and 1 pg for *seb* (data not shown).

A summary of data on the *S. aureus* strains used, including toxin phenotypes and observed PCR amplification products, appears in Table 1. The staphylococcal toxin-specific primers used in the PCR detected template NA only in strains of *S. aureus* found to produce enterotoxins A to E, ETA, ETB, or TSST-1 in biological assays. *S. aureus* strains characterized immunologically as SEC1- or SEC2-producing strains were both identified by the PCR with the SEC primers. The *S. aureus* ATCC strains included in the present study were confirmed by the PCR to be single-toxin producers, with the exception of ATCC 13565, which harbored both *sea* and *sed* genes. Primer specificity was determined in the PCR by using NA extracted from 26 strains of common human pathogens, including reference strains of *Vibrio cholerae*, *Shigella dysenteriae*, *Escherichia coli*, and *Streptococcus* spp. defined in terms of their toxigenicity. None of the recognized toxigenic pathogens tested in this study and previously described in some detail (23) contained templates capable of producing specific amplicons by the PCR with the staphylococcal toxin primers. In view of the close relationship of SPEA to both SEB and SEC1, five strains each of *Streptococcus pneumoniae*, *Streptococcus pyogenes* producing erythrogenic toxin, and *S. pyogenes* producing streptolysin O were included in the strains tested by the PCR, and no specific amplicons were observed.

Comparison of immunologically determined toxin phenotypes with their PCR-determined genotype. Equivocal results were obtained by RPLA tests for two of the 88 *S. aureus* strains tested immunologically for the production of toxins. By using the PCR with the eight pairs of oligonucleotide primers, gene sequences for SEC were clearly identified in one of these strains of *S. aureus* and gene sequences for TSST-1 were clearly identified in the other. Hence, equivocal results by the RPLA were unequivocal by the PCR for two strains of *S. aureus* carrying template for SEC or TSST-1, resulting in a concordance of 97.7% between the phenotypic and genotypic identifications. Complete correlation between assays for the gene product and the PCR were obtained for the remaining 86 strains of *S. aureus*, including 17 nontoxigenic isolates. Although 4 of the 17 negative strains had been previously identified as exfoliative toxin producers, neither ETA nor ETB could be detected immunologically, and the PCR failed to detect target gene sequences for the *eta* or *etb* genes. In addition, three of the ETA-producing *S. aureus* strains were previously identified as strains producing both ETA and ETB, although neither the *etb* gene nor ETB toxin could be demonstrated for these isolates by our tests.

During the present study, it was observed that the toxin phenotype determined in earlier toxin analyses did not match the current RPLA and PCR results for enterotoxins and TSST-1 in 29 of the 88 *S. aureus* strains. The most common mismatches were false-positive previously ascribed phenotypic recordings, and these occurred in strains of *S. aureus* purported to produce SEA (seven strains), SEB (seven strains), SEC (two strains), SED (three strains), and TSST-1 (three strains). False-negative records were recognized in strains subsequently identified by RPLA and the PCR as strains producing SEA (three strains), SEC (two strains), and SED (two strains).

DISCUSSION

We have designed and described rapid diagnostic protocols to detect genes for enterotoxins A to E, ETA, ETB, and TSST-1 in NA extracted from human and nonhuman strains of *S. aureus*. Although it is possible to detect the gene products immunologically by using a variety of enzyme-linked immunosorbent assays and radioimmunoassays (28), these tests have variable sensitivities and depend on adequate gene expression for reliability and reproducibility. Toxigenic strains of *S. aureus* with low levels of excreted toxin(s) or cross-reactive antigens could therefore be easily misidentified by immunologic methods. The eight pairs of synthetic oligonucleotide primers used individually in the PCR protocols described in this study target the structural genes for enterotoxins A to E (*sea*, *seb*, *sec-1*, *sed*, and *see*), ETA and ETB (*eta* and *etb*), and TSST-1 (*tst*) and specifically detected nucleotide sequences in template NA extracted from representative *S. aureus* strains. The primers were toxin-gene specific in that amplification was observed in the PCR only when NA template from strains of *S. aureus* characterized immunologically and biologically as toxigenic was used. No specific amplification was observed in the PCR by using template NA from clinical isolates of bacterial strains known to harbor virulence-associated toxins such as cholera toxin, verotoxins (VT1, VT2, and VTe), Shiga toxin,

and the classic heat-labile and heat-stable enterotoxins of *E. coli* (23). The presence or absence of a second nontarget toxin gene in NA from a single *S. aureus* isolate did not affect the detection of toxin-specific gene sequences by the PCR. *S. aureus* strains producing either SEC1 or SEC2 were identified by the SEC primers, reflecting the very high degree of homology (98%) among the three *sec* genes (*sec-1*, *sec-2*, and *sec-3*) (20).

Other methods for gene identification, such as DNA hybridization, have been used to analyze strains for the presence of staphylococcal toxin genes. Neill et al. (20) employed oligonucleotide probes to detect and differentiate *S. aureus* strains containing genes for SEA, SEB, SEC, and TSST-1 in colony blot hybridizations. In that study, a good correlation (≥93%) was observed between genotypic and phenotypic assays for SEA, SEC, and TSST-1. Other investigators have applied hybridization techniques using *seb* probes to detect both SEB and SEC (21), *sec-1* probes to identify SEB and SEC1 (6), *eta* and *etb* probes for ETA and ETB (24), and *tst* probes for TSST-1 (8). The detection limits observed in the staphylococcal toxin PCR protocol varied from one toxin gene to another and were 100 pg of total NA for *tst*, 10 pg for *sea*, *sec-1*, *sed*, *see*, *eta*, and *etb*, and 1 pg for *seb*. As a basis for comparison of sensitivities, a DNA oligonucleotide hybridization technique for detection of exfoliative toxins was reported to have a sensitivity of 10^6 bacteria or 100 ng of genomic DNA (24). The PCR protocol is therefore a far more sensitive genotypic technique than hybridization for identical target genes.

The synthetic oligonucleotide primers utilized in the staphylococcal toxin PCR application were designed to avoid areas of homology within the structural genes for the enterotoxins. Particular attention was focused on the primers for SEA and SEE since the *sea* and *see* genes were the most closely related, with an 84% nucleotide sequence similarity and no gaps in the alignment (9). In addition, primers for SEB and SEC1 were designed to avoid areas of close homology within the enterotoxin genes and also within *speA* (4, 19). Although the SPEA-derived amino acid sequence reported by Johnson et al. (14) for scarlet fever toxin had little homology with the staphylococcal enterotoxins, the nucleotide sequence reported by Weeks and Ferretti (30) for erythrogenic toxin and confirmed by Bohach and Schlievert (7), had 10 of 12 residues in common with three enterotoxins (19). A computer-assisted nucleotide sequence comparison was performed by using the published gene sequences for all the toxins in this study in addition to the *speA* (14, 30), *slo* (streptolysin O) (16), and *ply* (pneumolysin) (29) genes in order to identify potential binding sites for the primers within the streptococcal genes.

Computer analyses of the amino acid sequences showed that TSST-1 has little or no sequence homology with enterotoxins B and C1 or with SPEA (5, 19). The clinical strains of *S. aureus* identified phenotypically and genotypically as TSST-1 producers in the present study were subdivided into four groups on the basis of their toxin profiles. Of all the TSST-1 producing strains of *S. aureus* identified, 14% produced only TSST-1, 50% produced SEA and TSST-1, 25% produced SEC and TSST-1, and 11% produced SEB and TSST-1. These results established by immunologic and genotypic techniques confirm a previous report from this laboratory describing the simultaneous production of TSST-1 and SEB in suspected toxic shock syndrome isolates of *S. aureus* in Canada (12). Other laboratories (6, 20, 27) suggested that enterotoxin B and TSST-1 expression were mutually exclusive. The most common toxin profile observed in the TSST-1-producing strains was simultaneous SEA and TSST-1 production.

The *eta* and *etb* genes were clearly identified and distinguished by the PCR in 10 strains of *S. aureus* isolated from humans. ETA and ETB exhibit the same biological activity but are immunologically distinct toxins, and strains can produce one or both toxins. Alignment of the protein sequences of ETA and ETB indicated significant regions of homology at both the N and C terminus and in the middle of the toxins (17). The PCR application we describe offers the advantage of a rapid, specific, and inexpensive genotypic identification of strains harboring genes for ETA and ETB, with sensitivity an order of magnitude greater than that of hybridization techniques.

The presence or absence of the target staphylococcal toxin genes was determined definitively and unequivocally by the PCR for all of the strains tested in this study. Equivocal results were obtained by using the immunologic assays to determine toxin phenotypes in two strains of *S. aureus* identified by the PCR as carrying *sec* or *tst* genes. Some misidentification of strains held in the culture collection was observed and may be attributed to the lack of specificity and sensitivity for the toxins of assays performed several years ago, to poor expression of the genes, or to a lack of stability of the genetic elements carrying the toxin genes.

The value of genotypic methods in the direct detection of toxin-producing strains of *S. aureus* in pathological or food specimens has not yet been determined. The PCR has an advantage over DNA hybridization in that the sensitivity is sufficient to allow the detection of microbial DNA directly in pathological specimens (26). This genotypic technique, however, allows the detection of gene-harboring strains independent of their expression. Hence, a positive result in the PCR is indicative only of the presence of the targeted organism and does not indicate its viability or pathogenic toxic potential. The PCR may supplement enrichment protocols, which can often fail to detect virulent strains present at low levels in pathological or food samples. Frequently, nonpathogenic strains of the same genus or species overgrow the pathogens, and strains may readily lose plasmid- or phage-mediated virulence factors. The PCR allows specific enzymatic replication of targeted gene fragments, and since cell growth and replication are not required, both injured and viable cells will be detected and identified with equal facility. In foods, an indicator of dead cells yields valuable information as to the quality of foods but may not indicate a health hazard. The PCR can be performed by using whole bacterial cells without extraction of NA (22); coupled with preenrichment growth before the PCR, it dilutes out NA not being biologically duplicated and permits the identification of organisms in samples containing numbers of pathogenic bacteria undetectable by other routine methods.

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