

Multiplex PCR for Direct Detection of Shiga Toxigenic *Escherichia coli* Strains Producing the Novel Subtilase Cytotoxin

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Received 15 December 2004/Returned for modification 4 February 2005/Accepted 7 February 2005

We have recently described a novel AB₅ subtilase cytotoxin produced by certain Shiga toxigenic *Escherichia coli* (STEC) strains. This potentially lethal toxin may contribute to severe gastrointestinal and systemic disease in humans. In this study we have developed a trivalent PCR assay for the detection of the novel toxin A subunit gene *subA*, as well as *stx*₁ and *stx*₂. The three primer pairs used in the assay do not interfere with each other and generate amplification products of 556, 180, and 255 bp, respectively. The assay can be used for determining the toxin genotype of STEC isolates, as well as for direct detection of toxin genes in primary fecal culture extracts.

Shiga toxigenic *Escherichia coli* (STEC) is an important cause of gastrointestinal disease in humans, particularly since these infections may result in life-threatening sequelae such as the hemolytic-uremic syndrome (HUS) (7, 9, 18). STEC produces one or both of two major types of Shiga toxin, designated Stx1 and Stx2; production of the latter is associated with an increased risk of developing HUS (2, 8, 10). Other putative accessory virulence factors produced by subsets of STEC include the capacity to produce attaching and effacing lesions on intestinal mucosa, a property encoded by the locus for enterocyte effacement (LEE), as well as megaplasmid-encoded factors such as the enterohemolysin Ehx, the extracellular serine protease EspP, and the putative adhesin Saa (3, 4, 9, 18, 19).

While there is circumstantial evidence that the accessory virulence factors referred to above contribute to the pathogenesis of human gastrointestinal disease, the bulk of the pathology is believed to be directly attributable to the systemic effects of Stx, particularly damage to the microvascular endothelium (9, 18). However, we have recently reported that some STEC strains produce an additional, previously undescribed AB₅ cytotoxin, which is lethal for mice and also results in extensive microvascular damage, thrombosis, and necrosis in multiple organs, including the brain, kidneys, and liver (15). The new toxin has been named subtilase cytotoxin because its 35-kDa A subunit is a subtilase-like serine protease distantly related to the BA₂₈₇₅ gene product of *Bacillus anthracis*. The B subunit is related to a putative exported protein from *Yersinia pestis*, and like Stx, it forms a pentamer which mediates binding to glycolipid receptors on the target cell surface. The prototype Subtilase cytotoxin (SubAB) was detected in the LEE-negative O113:H21 STEC strain 98NK2, which was responsible for a small outbreak of HUS in Adelaide, South Australia, in 1998 (17). It is encoded by two closely linked, cotranscribed genes (*subA* and *subB*), which in 98NK2 are located on a large, conjugative virulence plasmid designated pO113 (15). The

overlapping pathology caused by SubAB in mice and Stx in humans and various animal models raises the intriguing question of whether the new toxin contributes to life-threatening disease in humans, either on its own or in concert with Stx.

Direct PCR analysis is increasingly being used for the detection of STEC in primary cultures of feces or foods (18). A positive reaction with primers specific for *stx*₁ or *stx*₂ is sufficient to confirm the presence of STEC in a sample, but use of primers capable of detecting accessory virulence genes (which are not universally distributed among STEC strains) provides additional clinically relevant information that may also be of great epidemiological value. Indeed, several multiplex PCR assays specific for *stx*₁, *stx*₂, various accessory virulence genes, and also some important STEC O or H serogroups have been described (11, 12, 13, 18). In view of the potential importance of SubAB in the pathogenesis of disease caused by STEC, we have designed a multiplex PCR assay for direct detection of the *subA* gene, as well as *stx*₁ and *stx*₂. This will facilitate screening of existing strain collections to determine the prevalence of the novel toxin gene in diverse STEC lineages, as well as in other *E. coli* pathotypes. It can also be used as a first-line screen for direct detection of strains carrying the toxin genes in clinical samples.

Multiplex toxin-specific PCR. Shiga toxin genes are highly variable, particularly within the *stx*₂ class (18), and our previously described diagnostic PCR primers for *stx*₁ and *stx*₂ were designed to react with all known sequence variants (11). Although we have shown by Southern hybridization analysis that homologues of *subAB* are present in a number of STEC strains in our collection (15), the extent of any sequence variation between these genes is currently unknown. In order to minimize the possibility of random sequence variations interfering with a diagnostic PCR, we designed primers specific for two of three critical functional domains in the SubA coding sequence. The three domains contain conserved Asp, His, and Ser residues, respectively, and collectively make up the “catalytic triad” characteristic of the subtilase family of serine proteases (15, 20). Primer SubHCDF (5′-TATGGCTTCCCTCATTCG C-3′) is specific for nucleotides 276 to 294 of the *subA* open reading frame, which encode part of the His catalytic domain,

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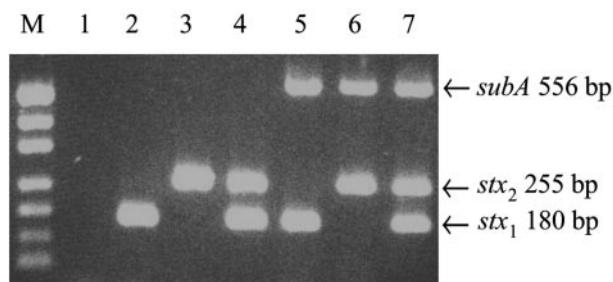


FIG. 1. PCR analysis of reference STEC strains. Lanes: M, DNA size markers (pUC19 DNA digested with HpaII; fragment sizes visible are 501/489, 404, 331, 242, 190, 147, and 111 bp); 1, negative control; 2 to 4, *subA*-negative STEC strains 98PY1, 99KN1, and 95AS1, respectively; 5 to 7, *subA*-positive STEC strains MW8, 98NK2, and 94CR, respectively. The expected mobilities of the *stx*₁-, *stx*₂-, and *subA*-specific PCR products are also indicated.

while primer SubSCDR (5'-TATAGCTGTTGCTTCTGACG-3') is complementary to nucleotides 812 to 831, which encode part of the Ser catalytic domain. These primers directed the amplification of a 556-bp fragment from the central region of *subA* using crude DNA from STEC strain 98NK2 as template, as predicted (result not shown). These were then combined with our previously described diagnostic PCR primers for *stx*₁ and *stx*₂ (11), which direct the amplification of 180- and 255-bp products, respectively.

The toxin-specific PCR was initially characterized using crude DNA extracts from 44 STEC strains in our collection. These strains had all been tested previously by PCR for *stx*₁ and *stx*₂, as well as for a number of additional putative accessory virulence factor genes. PCR templates were prepared by microfuging 1 ml of an overnight Luria-Bertani broth culture of each strain for 1 min. Pellets were resuspended in 75 μ l of sterile water, and 3 μ l of proteinase K (20 mg/ml) and 25 μ l of Chelex 100 (Bio-Rad Laboratories, Hercules, CA) (prepared as a 20% [wt/vol] suspension in sterile water) were then added. Tubes were vortexed and then incubated at 65°C for 60 min and then at 95°C for 20 min. Samples were stored at 4°C and microfuged for 3 min to pellet the Chelex resin immediately before withdrawing 3- μ l aliquots for use as PCR template. Samples were amplified by PCR in 50- μ l reaction mixtures containing 200 μ M deoxynucleoside triphosphates, approximately 250 nM each primer, and 1 U *Taq* polymerase (Roche Molecular Diagnostics, Germany) in 10 mM Tris-HCl (pH 8.3)–50 mM KCl–2 mM MgCl₂–0.1% gelatin–0.1% Tween 20–0.1% NP-40. Samples were subjected to 35 PCR cycles, each consisting of 1 min of denaturation at 95°C, 2 min of annealing at 65°C for the first 10 cycles, decrementing to 60°C by cycle 15, and 1.5 min of elongation at 72°C, incrementing to 2.5 min from cycles 25 to 35. PCRs were then electrophoresed on 2% agarose gels and stained with ethidium bromide.

Results for a selection of the tested strains are shown in Fig. 1, and collated data for all strains, including PCR results for other putative accessory virulence factor genes, are shown in Table 1. Sixteen of the 44 strains were *subA* positive, including representatives of serogroups O23, O82, O91, O113, and O148, as well as rough and O nontypeable strains. The results were consistent with previous data available for a subset of these strains, which had been tested for the presence of *subAB*-

TABLE 1. Presence of *subA* and other putative virulence genes in STEC strains^a

Strain	Serotype ^b	<i>subA</i>	<i>stx</i> ₁	<i>stx</i> ₂	<i>eae</i>	<i>ehxA</i>	<i>saa</i>	<i>pilS</i>
97MW1	O113:H21	+	–	+	–	+	+	+
98NK2	O113:H21	+	–	+	–	+	+	+
1183	O113:H21	+	–	+	–	+	+	+
3848	O113:H21	+	–	+	–	+	+	+
MW10	O113:H21	+	–	+	–	+	+	+
94CR	O148:H21	+	+	+	–	+	+	+
B2F1	O91:H21	–	–	+	–	+	+	–
95HE4	O91:H7	+	+	–	–	+	+	+
99AM1	O23	+	–	+	–	+	+	+
MW2	Ont	+	+	+	–	+	+	+
MW3	O82:H8	+	+	+	–	+	+	+
MW4	Ont	–	–	+	–	–	–	–
MW5	Ont	+	+	+	–	+	+	+
MW6	OR	+	+	+	–	+	+	+
MW7	Ont:H11	+	+	+	–	+	+	+
MW8	Ont	+	+	–	–	+	+	+
MW9	OR	+	+	+	–	+	+	+
MW11	ND ^c	–	+	+	–	–	–	–
MW12	O159	–	+	–	–	–	–	–
MW13	O98	–	–	+	–	+	+	+
MW14	ND	+	+	–	–	+	+	+
95AS1	O128	–	+	+	–	–	–	–
95MV1	OR:H9	–	–	+	–	–	–	–
99KN1	ND	–	–	+	–	–	–	–
031	OX3:H21	–	–	+	–	–	–	–
DG133/2	OX3:H8	–	+	+	–	–	–	–
DG155/2	OX3:H8	–	+	+	–	+	–	+
DG166/2	OX3:H8	–	+	+	–	+	–	+
95ZG1	O26:H11	–	+	–	+	+	–	–
95PY1	O26	–	+	–	+	+	–	–
94AS1	O111	–	+	+	+	+	–	–
CB168	O111	–	+	–	+	+	–	–
CB779	O111	–	+	–	+	+	–	+
CB533	O111	–	+	–	+	+	–	+
95JS1	O111:H [–]	–	+	+	+	+	–	–
PH	O111:H [–]	–	+	+	+	+	–	–
98GE1	O111	–	+	+	+	+	–	–
99BG1	O111	–	+	+	+	+	–	–
J26752	O111	–	+	+	+	+	–	–
95DB1	O111:H [–]	–	+	+	+	+	–	–
95SF1	O111:H [–]	–	+	+	+	+	–	–
96GR1	O157:H [–]	–	–	+	+	+	–	–
95FE1	O157:H [–]	–	–	+	+	+	–	–
99SK1	O157	–	–	+	+	+	–	–

^a Genotypes determined by PCR, as previously described (13, 21).

^b OR, O rough; Ont, O non-typable.

^c ND, not determined.

related sequences by Southern hybridization analysis of purified genomic DNA (15). All those that were previously negative by hybridization were also negative by PCR for *subA*, whereas those which yielded a strong hybridization signal at high stringency were all PCR positive. A small number of strains belonging to serogroups O111 and O157, which had previously yielded weak *subAB* hybridization signals (15), were negative by PCR. In an attempt to resolve this apparent discrepancy, the probe-reactive DNA fragment from one such strain (95NR1) was cloned into *E. coli* JM109 using pBlue-script. Sequence analysis indicated that this fragment contained a region with partial homology to *subAB*, but it did not contain a complete copy of the operon. Thus, the PCR assay provided 100% specificity. All of the *subA*-positive strains were also positive for the enterohemolysin gene *ehxA*, which is com-

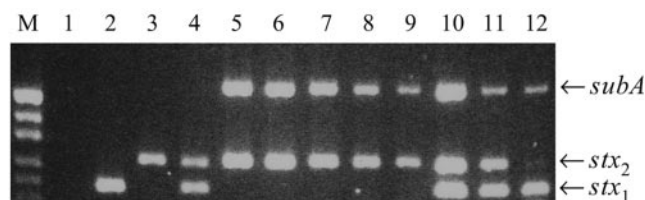


FIG. 2. Multiplex PCR analysis of crude DNA extracts from primary fecal cultures. Lanes: M, DNA size markers (as for Fig. 1); 1, negative control; 2 to 12, extracts from patients with culture-proven STEC infections. The expected mobilities for the various PCR products are also indicated.

monly used as a marker for the presence of a megaplasmid. This is consistent with the proven location of the *subAB* operon on the megaplasmid pO113 in the prototype strain 98NK2 (15). In 98NK2/pO113, *subAB* is flanked at the 3' end by the autoagglutinating adhesin gene *saa* (16), while a type IV pilus locus which includes the *pilS* gene (21) is located approximately 7 kb upstream (15). All 16 of the *subA*-positive STEC strains tested were also positive for both *saa* and *pilS*. However, the linkage was not 100%, as one strain which was positive for both flanking genes was negative for *subA*, while a further six *subA*-negative strains were positive for either *saa* or *pilS*.

Multiplex PCR analysis of primary fecal culture extracts.

To demonstrate the diagnostic utility of the multiplex assay, crude DNA extracts of primary fecal cultures (prepared as described above) from 171 patients with diarrhea were tested. Each of these samples had previously tested positive by PCR for the presence of the *stx*₁ and/or *stx*₂ genes, confirming the diagnosis of STEC infection. These 171 samples (stored at -15°C) were collected over approximately 4 years and represent all *stx*-positive cases from South Australia for which sufficient residual DNA extract was available. Multiplex PCR results for a subset of these samples are shown in Fig. 2, while collated data for all samples are shown in Table 2. Analysis of crude fecal culture extracts from 12 healthy controls yielded negative results for all target genes (result not shown). Nineteen (11%) of the 171 *stx*-positive samples were positive for *subA*. Interestingly, 41.7% of samples that were positive for *stx*₂ only were also *subA* positive, compared with only 2.9% of those with *stx*₁ alone and 3.7% of those with both *stx* genes.

Discussion and conclusions. PCR is increasingly being used for rapid diagnosis of enteric infections, particularly those caused by pathogenic *E. coli* strains that are not easily distinguished from commensal organisms by simple culture techniques. Although direct extracts of feces or foods can be used

as templates for PCR, the best results are usually obtained by testing extracts of primary broth cultures (1, 5, 14, 18). The broth enrichment step can involve as little as 4 h of incubation and serves two purposes: inhibitors in the sample are diluted, and bacterial growth increases the number of copies of the target sequence, enhancing sensitivity. For STEC infections, detection of either the *stx*₁ or the *stx*₂ gene confirms the diagnosis but testing for the presence of additional gene sequences can provide clinically and epidemiologically important additional information about the infecting strain(s), even under circumstances in which isolates are not obtained. For example, testing for *eae* and *ehxA* confirms the presence of the LEE pathogenicity island and the large virulence plasmid, respectively, both of which are more commonly found in STEC strains associated with severe human disease (2, 6, 19).

The recent discovery that some STEC strains produce a potent AB₅ cytotoxin (SubAB) that has the potential to significantly augment clinical manifestations, or indeed to cause disease in its own right, raises several important questions. For example, what is the prevalence of SubAB-producing STEC strains? How widely distributed are the *subAB* genes among disease-causing STEC strains? Are the genes found only in certain STEC lineages? Is SubAB produced by any non-STEC strains, and most importantly, is there any association between production of SubAB and severity of STEC disease in humans? Answering these questions will require analysis of much larger strain collections, a task for which PCR is well suited. In the present study, we have designed PCR primers based on structurally constrained regions of the *subA* gene, which are likely to be highly conserved, and combined these with primers specific for *stx*₁ and *stx*₂ in a multiplex format. The various primers were designed such that the PCR products differ in size (180, 255, and 556 bp) and so can be readily distinguished by agarose gel electrophoresis. We have demonstrated the utility of the trivalent PCR for characterization of individual isolates, thereby facilitating screening of existing STEC strain collections from diverse geographic regions. We have also used it to test 171 crude DNA extracts from primary fecal cultures from patients with proven STEC disease and demonstrated that 11% of these samples were also *subA* positive. We also demonstrated a strong association between the presence of *subA* and STEC carrying *stx*₂ only; this STEC subset has already been associated with an increased risk of serious human disease, such as HUS. Although not examined in the present study, it is possible that SubAB is produced by other (non-STEC) pathogenic *E. coli* strains. This possibility could be addressed by using the multiplex PCR assay described herein as a primary diagnostic screening method.

This work was supported by program grant 284214 from the National Health and Medical Research Council of Australia.

TABLE 2. Presence of *subA* in *stx*-positive crude fecal culture extracts

<i>stx</i> genotype	No. of samples	
	<i>subA</i> positive	<i>subA</i> negative
<i>stx</i> ₁ positive, <i>stx</i> ₂ negative	1	34
<i>stx</i> ₁ negative, <i>stx</i> ₂ positive	15	36
<i>stx</i> ₁ positive, <i>stx</i> ₂ positive	3	82
Total	19	152

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