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Detection and Characterization of Shiga Toxigenic *Escherichia coli* by Using Multiplex PCR Assays for stx_1, stx_2, *eaeA*, Enterohemorrhagic *E. coli hlyA*, rfb_{O111}, and rfb_{O157}

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Shiga toxigenic *Escherichia coli* (STEC) comprises a diverse group of organisms capable of causing severe gastrointestinal disease in humans. Within the STEC family, certain strains appear to be of greater virulence for humans, for example, those belonging to serogroups O111 and O157 and those with particular combinations of other putative virulence traits. We have developed two multiplex PCR assays for the detection and genetic characterization of STEC in cultures of feces or foodstuffs. Assay 1 utilizes four PCR primer pairs and detects the presence of stx_1, stx_2 (including variants of stx_2), *eaeA*, and enterohemorrhagic *E. coli hlyA*, generating amplification products of 180, 255, 384, and 534 bp, respectively. Assay 2 uses two primer pairs specific for portions of the *rfb* (O-antigen-encoding) regions of *E. coli* serotypes O157 and O111, generating PCR products of 259 and 406 bp, respectively. The two assays were validated by testing 52 previously characterized STEC strains and observing 100% agreement with previous results. Moreover, assay 2 did not give a false-positive O157 reaction with enteropathogenic *E. coli* strains belonging to clonally related serogroup O55. Assays 1 and 2 detected STEC of the appropriate genotype in primary fecal cultures from five patients with hemolytic-uremic syndrome and three with bloody diarrhea. Thirty-one other primary fecal cultures from patients without evidence of STEC infection were negative.

Shiga toxigenic *Escherichia coli* (STEC) is an important cause of gastrointestinal disease in humans, particularly since such infections may result in life-threatening sequelae such as hemolytic-uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (22). The morbidity and mortality associated with several recent large outbreaks of STEC disease have highlighted the threat these organisms pose to public health (17, 25, 28, 32). For this reason, there is an increasing demand for improved diagnostic procedures for the detection of STEC in fecal samples and, in particular, in foods such as meat and dairy products. It has been recognized for a number of years that STEC strains causing human disease may belong to a very broad range of O serogroups (22). However, many of the STEC strains found in the gastrointestinal tracts of domestic animals (the principal source of human infections) may have a low degree of virulence in humans. These strains are less likely to produce putative accessory virulence factors such as intimin (encoded by *eaeA*) and the plasmid-encoded enterohemolysin (encoded by enterohemorrhagic *E. coli* (EHEC) *hlyA*) (1, 5, 6, 33, 34). Within the human disease-associated strains, those producing Shiga toxin type 2 (Stx2, encoded by stx_2) appear to be more commonly responsible for serious complications such as HUS than those producing only Shiga toxin type 1 (Stx1, encoded by stx_1) (23, 26). Furthermore, STEC belonging to serogroup O157 and, to a lesser extent, serogroup O111 are responsible for the vast majority of HUS outbreaks (9, 16, 25, 28, 32). Accordingly, the capacity to rapidly determine whether a patient is infected, or food is contaminated, with STEC belonging to serogroup O111 or O157 or whether the STEC produces virulence factors associated with more serious disease would be highly advantageous. For this reason, we have developed multiplex PCR assays for the simultaneous detection of (i) stx_1, stx_2, *eaeA*, and EHEC *hlyA* and (ii) portions of the *rfb* (O-antigen-encoding) regions of *E. coli* O111 and O157.

Examination of STEC isolates by multiplex PCR. PCR primer pairs were designed with reference to published sequence data for stx_1 (19), stx_2 (18), *eaeA* (35), EHEC *hlyA* (33), and portions of the *rfb* regions of *E. coli* O111 (2) and O157 (7). Details of the nucleotide sequence, the specific gene region amplified, and the size of the PCR product for each primer pair are listed in Table 1. Crude DNA extracts were prepared from characterized STEC strains, as described previously (27). A total of 52 STEC strains were examined, and these were from our own collection or were kindly provided by Roy Robins-Browne, Royal Children's Hospital, Melbourne, Australia, or Lothar Beutin, Robert Koch Institute, Berlin, Germany. Twenty eight strains were isolated from human feces (patients with diarrhea or HUS), 7 were from domestic animals, and 17 were from foods. The STEC serogroups included O157 (19 isolates), O111 (10 isolates), O26 and OX3 (2 isolates each), and O48, O91, O98, O113, O128, O141, and O159 (1 isolate each); a further 12 STEC isolates were not typeable by the O serogroup. Four *stx*-negative enteropathogenic *E. coli* (EPEC) strains belonging to serogroups O55 (three isolates provided by R. Robins-Browne) and O111 (one isolate) and a sorbitol-fermenting, nontoxigenic O157:H20 isolate (also provided by R. Robins-Browne) were also tested.

Samples (2 μl) of each extract were amplified in 50-μl reaction mixtures containing 200 μM concentrations of deoxynucleoside triphosphates, an approximately 250 nM concentration of each primer, and 1 U of *Taq* polymerase (Boehringer GmbH, Mannheim, Germany) in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.1% gelatin, 0.1% Tween 20, and 0.1% Nonidet P-40. Samples were subjected to 35 PCR cycles, each consisting of 1 min of denaturation at

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TABLE 1. PCR primers

Primer	Sequence (5′–3′)	Specificity[a]	Amplicon size (bp)
Assay 1			
stx1F	ATAAATCGCCATTCGTTGACTAC	nt 454–633 of A subunit coding region of stx_1	180
stx1R	AGAACGCCCACTGAGATCATC		
stx2F	GGCACTGTCTGAAACTGCTCC	nt 603–857 of A subunit coding region of stx_2 (including stx_2 variants)	255
stx2R	TCGCCAGTTATCTGACATTCTG		
eaeAF	GACCCGGCACAAGCATAAGC	nt 27–410 of *eaeA* (this region is conserved between EPEC and STEC)	384
eaeAR	CCACCTGCAGCAACAAGAGG		
hlyAF	GCATCATCAAGCGTACGTTCC	nt 70–603 of EHEC *hlyA*	534
hlyAR	AATGAGCCAAGCTGGTTAAGCT		
Assay 2			
O157F	CGGACATCCATGTGATATGG	nt 393–651 of $rfbE_{O157:H7}$	259
O157R	TTGCCTATGTACAGCTAATCC		
O111F	TAGAGAAATTATCAAGTTAGTTCC	nt 24–429 of ORF 3.4 of *E. coli* O111 *rfb* region	406
O111R	ATAGTTATGAACATCTTGTTTAGC		

[a] nt, nucleotide; ORF, open reading frame.

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. PCR reaction mixtures were electrophoresed on 2% agarose gels and stained with ethidium bromide. Figure 1 shows a representative gel for eight previously characterized STEC strains subjected to PCR assay 1. Clear PCR products of the expected sizes were observed, consistent with the presence of stx_1, stx_2, *eaeA*, and EHEC *hlyA*. Assay 1 PCR results for all 52 STEC strains tested are shown in Table 2. Again, there was 100% agreement with previously determined genotype data (28, 29).

Figure 2 shows a representative gel for eight reference STEC isolates (four O111 strains and four O157 strains), as well as for an O55 EPEC isolate (which is genetically related to O157:H7 STEC strains), analyzed by PCR assay 2. Clear PCR products of the expected sizes were seen for each of the O111 and O157 strains (406 bp and 259 bp, respectively), but the O55 strain did not yield a PCR product. An analysis of the entire STEC collection showed that all 10 O111 strains tested as well as O111 EPEC strain 87A yielded a 406-bp PCR product. The 19 O157 STEC strains tested (including O157:H$^-$ and O157:H7 strains) all yielded a 259-bp PCR product, as did the sorbitol-fermenting, nontoxigenic O157:H20 isolate. The 23 STEC isolates tested that did not belong to either serogroup O111 or O157 yielded no PCR products (result not presented). All three O55 EPEC isolates tested were negative by assay 2, although they did give a positive PCR result for *eaeA* by assay 1 (as did O111 EPEC strain 87A) (result not presented).

Analysis of primary fecal cultures by multiplex PCR. To demonstrate the diagnostic utility of multiplex PCR, crude DNA extracts of primary fecal cultures from four sporadic HUS cases and one patient with bloody diarrhea were analyzed by assays 1 and 2 (Fig. 3). PCR analysis indicated that fecal cultures from HUS patients 1 and 2 contained O111 STEC strains which were positive for stx_1, stx_2, *eaeA*, and EHEC *hlyA*. The fecal culture from HUS patient 3 contained a STEC isolate not belonging to serogroup O111 or O157 which was positive for stx_1, stx_2, and EHEC *hlyA* but which was negative for *eaeA*, while HUS patient 4 had an O157 STEC isolate which was positive for stx_2, *eaeA*, and EHEC *hlyA*. The fecal culture from patient 5, who had bloody diarrhea, contained an O157 STEC isolate which was positive for stx_1, stx_2, *eaeA*, and EHEC *hlyA*. Fecal cultures from a further three cases of STEC infection were also tested (results not presented). These included one HUS case caused by an O157 STEC isolate positive for stx_2, *eaeA*, and EHEC *hlyA*, one bloody diarrhea case caused by an STEC isolate not of serogroup O111 or O157 that

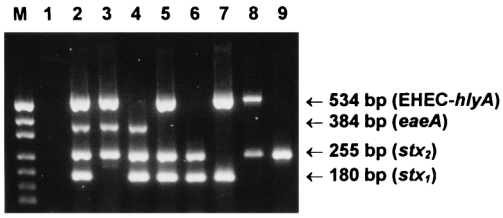

FIG. 1. Characterization of reference STEC strains by multiplex PCR assay 1. Lanes: M, DNA size markers (pUC19 DNA digested with *Hpa*II; fragment sizes visible are 501/489, 404, 331, 242, 190, 147, and 111 bp); 1, negative control; 2, O157:H$^-$ strain 96/2998 (stx_1^+ stx_2^+ *eaeA*$^+$ EHEC *hlyA*$^+$); 3, O157 strain 94-8628 (stx_2^+ *eaeA*$^+$ EHEC *hlyA*$^+$); 4, O157 strain 96/0052 (stx_1^+ stx_2^+ *eaeA*$^+$); 5, O48:H21 strain 94CR (stx_1^+ stx_2^+ EHEC *hlyA*$^+$); 6, O128 strain 95AS1 (stx_1^+ stx_2^+); 7, O91 strain 95HE4 (stx_1^+ EHEC *hlyA*$^+$); 8, O113 strain MW10 (stx_2^+ EHEC *hlyA*$^+$); 9, OX3:H21 strain O31 (stx_2^+). The expected mobilities for the various specific PCR products are also indicated.

TABLE 2. Characterization of STEC by multiplex PCR

STEC genotype[a]				No. of isolates by source			
stx_1	stx_2	*eaeA*	*hlyA*	Human	Animal	Food	Total
+	−	−	−	0	0	2	2
−	+	−	−	1	0	2	3
+	+	−	−	3	1	1	5
+	−	−	+	1	0	2	3
−	+	−	+	0	0	2	2
+	+	−	+	1	0	6	7
+	−	+	−	0	0	0	0
−	+	+	−	0	0	0	0
+	+	+	−	1	0	0	1
+	−	+	+	6	0	0	6
−	+	+	+	6	1	2	9
+	+	+	+	9	5	0	14

[a] +, gene present; −, gene absent.

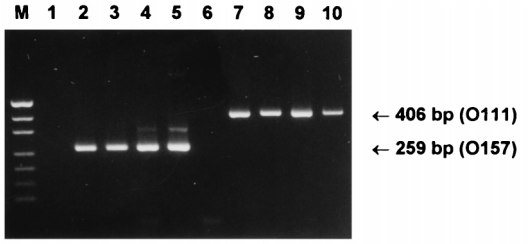

FIG. 2. Characterization of reference STEC strains by multiplex PCR assay 2. Lanes: M, DNA size markers (pUC19 DNA digested with *Hpa*II; fragment sizes visible are 501/489, 404, 331, 242, 190, 147, and 111 bp); 1, negative control; 2, O157:H$^-$ strain 95SF2; 3, O157:H$^-$ strain 96GR1; 4, O157 strain 96/0052; 5, O157:H7 strain 90/103; 6, O55 EPEC strain 93/282; 7, O111:H$^-$ strain 95NR1; 8, O111:H$^-$ strain 96RO1; 9, O111:H$^-$ strain PH; 10, O111:H$^-$ strain CB168. The expected mobilities for the O111- and O157-specific PCR products are also indicated.

was positive for stx_2, *eaeA*, and EHEC *hlyA*, and one bloody diarrhea case complicated with microangiopathic hemolytic anemia and thrombocytopenia caused by an O113 STEC isolate positive for stx_2 and EHEC *hlyA*. In all eight cases, the direct PCR results were 100% compatible with the genotype of the STEC which was isolated from the cultures. A total of 32 other primary cultures of fecal samples submitted for bacteriological investigation were also tested (results for two of which [patients 6 and 7] are also shown in Fig. 3). Three of these samples had yielded *Salmonella sp.*, one grew *Shigella flexneri*, and one was positive for rotavirus; the remainder did not yield a pathogen. Thirty one of these samples were negative by both assays 1 and 2 and were also negative when tested by an independent *stx*-specific PCR assay (27). The remaining sample (from a patient who had diarrhea but was negative for other pathogens) yielded a very weak positive stx_2 reaction by assay 1, which was subsequently confirmed by the independent *stx*-specific PCR assay (27), but no PCR products were seen by assay 2. Isolation of STEC from this culture was not attempted, as the weak PCR signal suggested that only very small numbers of STEC would have been present.

Sensitivity of multiplex PCR. To assess sensitivity, one of the STEC-negative fecal cultures tested above was spiked with serial dilutions of cultures of STEC strains 95NR1 (O111:H$^-$) or 95SF2 (O157:H$^-$). Extracts of these samples were then subjected to assay 2 (Fig. 4). For both the O111 and the O157 serogroup strains, a PCR product of the appropriate size could still be seen in the sample that contained the 10^4-fold-diluted STEC culture (equivalent to approximately 10^3 STEC CFU per assay), but not in the sample containing 10^5-fold dilutions. Also, the presence of both O111 and O157 STEC (each diluted 10-fold) in a sample did not appear to interfere with the strength of the PCR signal (Fig. 4).

Discussion and conclusions. PCR is generally considered to be the most sensitive means of determining whether a fecal specimen or a food sample contains STEC. 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 (3, 14, 27). Broth enrichment serves two purposes: inhibitors in the sample are diluted, and bacterial growth increases the number of copies of the target sequence. Sensitivity is important when testing fecal samples, because although STEC numbers may be very high in the early stages of infection, they may drop dramatically as disease progresses. Sensitivity is also particularly important when testing suspected foods because, at least for certain O111 and O157 STEC strains, the infectious dose for humans may be of the order of 1 to 10 CFU (17, 28). Thus, foods destined to be consumed without further cooking or processing need to be totally free of such STEC strains. Some of the PCR assays for detection of STEC described to date use single pairs of primers based on consensus sequences, which are capable of amplifying all *stx*-related genes, with subsequent identification of the *stx* type requiring Southern or dot blot hybridization with labelled oligonucleotides directed against type-specific sequences within the amplified fragment (21, 27, 31). Others combine different primer pairs for stx_1 and stx_2, and in some cases stx_2 variants, in the same reaction and direct the amplification of fragments which differ in size for each toxin type (4, 8, 14, 20, 30).

PCR has also been used for the detection of genes encoding accessory STEC virulence factors, such as *eaeA* and EHEC *hlyA* (15, 33). Fratamico et al. (12) combined previously described *stx*- (21) and *eaeA*-specific (15) PCR primer pairs with those specific for a portion of the 60-MDa virulence plasmid from an O157:H7 STEC in a multiplex assay. They concluded that this assay was suitable for the identification of STEC belonging to serogroup O157. However, the O157 virulence plasmid primers used actually recognize a portion of the EHEC *hlyA* gene, which of course is not confined to serogroup O157. Gannon et al. (13) have recently described two other multiplex PCR formats based on a *stx*-specific primer pair and two distinct *eaeA*-specific primer pairs, as well as a primer pair specific for a portion of the $fliC_{h7}$ gene, which encodes the H7 antigen. The two *eaeA*-specific primer sets recognized either the highly conserved 5′ portion of *eaeA* or a region at the (variable) 3′ end of the gene which was specific for O157 strains and a small number of other serogroups, including O55 (15). A further multiplex PCR has been used to detect O157:H7 STEC; the PCR uses stx_1- and stx_2-specific primer pairs in combination with a primer pair that recognizes a single base mutation in the β-glucoronidase-encoding *uidA* gene (10). This mutation in *uidA* results in a β-glucoronidase-negative phenotype, a feature strongly (although not absolutely) associated with O157:H7 strains. Louie et al. (24) also ex-

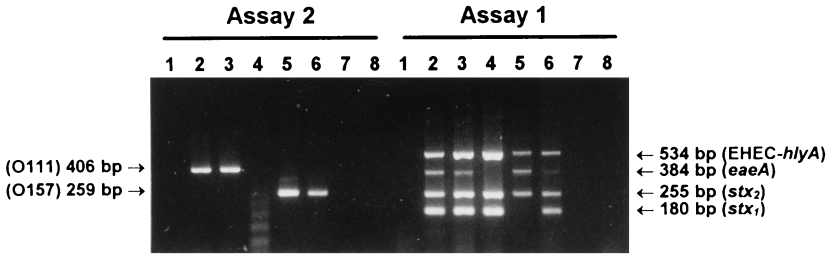

FIG. 3. Multiplex PCR analysis of primary fecal cultures. Crude DNA extracts of primary fecal cultures were analyzed by multiplex PCR assay 1 or assay 2, as indicated. Lanes: 1, negative control; 2, patient 1 (HUS); 3, patient 2 (HUS); 4, patient 3 (HUS); 5, patient 4 (HUS); 6, patient 5 (bloody diarrhea); 7, patient 6 (control); 8, patient 7 (control). The expected mobilities for the various specific PCR products are also indicated.

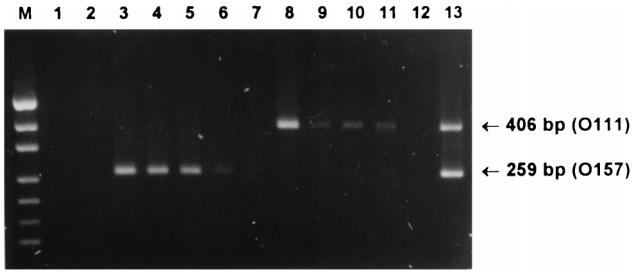

FIG. 4. Sensitivity of multiplex PCR assay 2. An STEC-negative fecal culture was spiked with serial 10-fold dilutions of cultures of STEC strains 95NR1 (O111:H$^-$) or 95SF2 (O157:H$^-$). Extracts of these samples were then subjected to assay 2. Lanes: M, DNA size markers (pUC19 DNA digested with *Hpa*II; fragment sizes visible are 501/489, 404, 331, 242, 190, 147, and 111 bp); 1, negative control; 2, STEC-negative fecal culture (FC); 3, FC plus a 10-fold dilution of a strain 95SF2 culture (10^{-1} 95SF2); 4, FC plus 10^{-2} 95SF2; 5, FC plus 10^{-3} 95SF2; 6, FC plus 10^{-4} 95SF2; 7, FC plus 10^{-5} 95SF2; 8, FC plus 10^{-1} 95NR1; 9, FC plus 10^{-2} 95NR1; 10, FC plus 10^{-3} 95NR1; 11, FC plus 10^{-4} 95NR1; 12, FC plus 10^{-5} 95NR1; 13, FC plus 10^{-1} 95SF2 plus 10^{-1} 95NR1. The expected mobilities for the O111- and O157-specific PCR products are also indicated.

ploited the heterogeneity of the 3′ end of *eaeA* to design two PCR assays, one of which was specific for *eaeA* from O157 STEC strains and O55 EPEC strains. The other primer pair gave a positive reaction with 16 of 22 O111 STEC strains but not with O111 EPEC strains. The sequence heterogeneity of *eaeA* within O111 STEC is presumably responsible for the fact that six of the strains (all of which were O111:H11) were found to be negative by PCR even though they hybridized to a probe specific for the conserved region of *eaeA*.

In the present study, we have designed two multiplex PCR assays for the detection and characterization of STEC. Assay 1 detects the presence of stx_1, stx_2, *eaeA*, and EHEC *hlyA*, generating PCR products of distinct sizes which are easily distinguished after agarose gel electrophoresis. Mismatch between the primer and target sequences due to the known heterogeneity of stx_2 genes has the potential to interfere with PCR assays, but the primers used in the present study were designed to amplify all stx_2 variants currently deposited with GenBank. Similarly, the *eaeA* primers are specific for a region at the 5′ end of the gene that appears to be conserved among all STEC and EPEC strains examined to date. Moreover, the PCR primers for the amplification of EHEC *hlyA* were designed to eliminate the possibility of cross-reaction with the gene encoding *E. coli* alpha-hemolysin, with which it shares about 70% DNA sequence homology. Multiplex PCR assay 2 detects the presence of genes involved in the biosynthesis of serogroup O111 and O157 O antigen. The actual region of the O111 *rfb* gene cluster (open reading frame 3.4) that is amplified is similar to that described previously (28), but the primer sequences have been modified such that their melting temperatures are similar to those for the primers designed to amplify the O157 *rfbE* gene. These primers are the only ones described to date which directly target O-antigen-encoding genes, and the assay is not dependent on clonal association between particular variants of unrelated genes (e.g., *eaeA* or *uidA*) and STEC strains of a given serotype. Thus, all O111 and O157 strains tested positive, and for the O157-specific PCR, there was no reaction with clonally related EPEC strains belonging to serogroup O55.

The two multiplex PCR assays were also found to be effective for the direct detection and characterization of STEC in primary fecal cultures from eight patients with HUS or bloody diarrhea. The absence of reactivity of fecal cultures from persons without evidence of STEC infection (including those known to be infected with other pathogens) is further evidence for the specificity of both assay 1 and assay 2. Information on the genotype of a STEC isolate may be of considerable microbiological and clinical significance because, as discussed previously, there appears to be a link between certain combinations of traits and the capacity of a STEC isolate to cause serious gastrointestinal disease in humans and complications such as HUS. The presence of these particular STEC genotypes in a food source (e.g., meat) is also extremely significant from a food safety or public health viewpoint. Previous studies have shown that PCR assays based on *stx* sequences can detect the presence of very low numbers of STEC organisms in microbiologically complex samples (8, 27, 28). The analysis of spiked fecal cultures in the present study demonstrated that serogroup-specific PCR assay 2 was also very sensitive. Fecal cultures spiked with 10^4-fold dilutions of O111 or O157 STEC cultures (i.e., the STEC constituted 0.01% of the total flora) generated a PCR product which was visible on an ethidium bromide-stained agarose gel. Other direct methods (e.g., plating on cefixime-tellurite sorbitol MacConkey agar or latex agglutination for O157 STEC, enzyme-linked immunosorbent assay for either lipopolysaccharide or Stx, etc.) are unlikely to detect such low levels of STEC. The sensitivity of the PCR could undoubtedly be improved even further by increasing the volume of sample assayed or by secondary hybridization of the PCR products with labelled *rfb*-specific probes.

Although much useful information can be gained from multiplex PCR analysis of fecal or food samples, the interpretation is complicated by the possibility that the composite genotypic profile may represent the sum of genotypes of more than one STEC organism. For this reason, isolation of STEC from plate cultures, for example by colony hybridization with *stx*-specific probes, should always be attempted. However, given the sensitivity of PCR screens, there is a likelihood that a proportion of genuine STEC PCR-positive specimens will not yield an isolate even after heroic efforts. The probability of isolating STEC is considerably greater, however, if the infecting serogroup is known, as this permits the deployment of immunomagnetic bead enrichment techniques, which have already been described for serogroups O157 (11) and O111 (28). The capacity to target these procedures at specimens that have been proven by multiplex PCR to contain STEC belonging to these serogroups should result in significant savings in terms of labor.

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