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Detection in *Escherichia coli* of the Genes Encoding the Major Virulence Factors, the Genes Defining the O157:H7 Serotype, and Components of the Type 2 Shiga Toxin Family by Multiplex PCR

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Strains of Shiga toxin-producing *Escherichia coli* (STEC) have been associated with outbreaks of diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome in humans. Most clinical signs of disease arise as a consequence of the production of Shiga toxin 1 (Stx1), Stx2 or combinations of these toxins. Other major virulence factors include enterohemorrhagic *E. coli* hemolysin (EHEC *hlyA*), and intimin, the product of the *eaeA* gene that is involved in the attaching and effacing adherence phenotype. In this study, a series of multiplex-PCR assays were developed to detect the eight most-important *E. coli* genes associated with virulence, two that define the serotype and therefore the identity of the organism, and a built-in gene detection control. Those genes detected were stx_1, stx_2, stx_{2c}, stx_{2d}, stx_{2e}, stx_{2f}, EHEC *hlyA*, and *eaeA*, as well as *rfbE*, which encodes the *E. coli* O157 serotype; *fliC*, which encodes the *E. coli* flagellum H7 serotype; and the *E. coli* 16S rRNA, which was included as an internal control. A total of 129 *E. coli* strains, including 81 that were O157:H7, 10 that were O157:non-H7, and 38 that were non-O157 isolates, were investigated. Among the 129 samples, 101 (78.3%) were *stx* positive, while 28 (21.7%) were lacked *stx*. Of these 129 isolates, 92 (71.3%) were EHEC *hlyA* positive and 96 (74.4%) were *eaeA* positive. All STEC strains were identified by this procedure. In addition, all Stx2 subtypes, which had been initially identified by PCR-restriction fragment length polymorphism, were identified by this method. A particular strength of the assay was the identification of these 11 genes without the need to use restriction enzyme digestion. The proposed method is a simple, reliable, and rapid procedure that can detect the major virulence factors of *E. coli* while differentiating O157:H7 from non-O157 isolates.

Many strains of *Escherichia coli* produce a variety of potent toxins, including Shiga-like toxins (Stx). Indeed it is often by virtue of these toxins that Shiga toxin producing *E. coli* (STEC) isolates express virulence for humans (10, 12). These strains are often referred to as verotoxin-producing *E. coli* due to the effect these toxins have on Vero cells in culture (12, 15). Such toxigenic isolates have been identified as a worldwide cause of serious human gastrointestinal disease, often with severe complicating problems that include bloody diarrhea, hemorrhagic colitis (HC), and the life-threatening condition hemolytic-uremic syndrome (HUS) (10).

Using in vitro and animal model studies, several groups have reported that a number of factors account for the virulence of STEC isolates, and prominent among these is the Stx group of toxins (4, 12, 15). Based on serological methods and DNA sequence analysis, these Stx toxins have been divided in to two major subclasses, Stx1 and Stx2 (16; S. C. Head, M. A. Karmali, M. E. Roscoe, M. Petric, N. A. Strockbine, and I. K. Wachsmuth, Letter, Lancet **ii:**751, 1988). Since there is no currently available specific treatment for HUS, there is an urgent need for effective preventive measures based on a detailed understanding of the epidemiology of STEC infections. Such measures will also be dependent on the availability of rapid, sensitive, simple, and reproducible procedures for the detection of these pathogens and for the characterization of their toxins both in samples from human specimens and those of nonhuman origin such as food and water.

Although Stx1 is relatively homogeneous, five subtypes of Stx2 have been identified, and these include Stx2, Stx2c (15, 20), Stx2d (27, 28, 31), Stx2e (11, 21), and Stx2f (7, 35). Indeed, based on both restriction fragment length polymorphisms (RFLP) of the B-subunit encoding the DNA fragments obtained by PCR and digoxigenin oligonucleotide labeling, DNA probes specific for stx_2 B-subunit genes encoding Stx2, Stx2c, and Stx2d have been further confirmed (4, 6, 8, 9, 14, 15, 24–26, 31, 37, 38). Other major virulence factors ascribed to the pathogen include a plasmid-encoded enterohemolysin from STEC (enterohemorrhagic *E. coli* [EHEC] *hlyA*) that is often associated with severe clinical disease in humans (33, 34) as well as intimin, the product of the *eaeA* gene involved in the bacterial attaching and effacing adherence phenomenon and clustered in a pathogenicity island termed the locus for enterocyte effacement (22, 23). In addition, the presence of the *ehl1* gene, which encodes an enterohemolysin that is unrelated to the EHEC *hlyA* genotype, has been associated with a severe outbreak of *E. coli* disease among neonates (2).

For detection purposes, a number of multiplex-PCR assays have been developed for the various virulence genes associated with *E. coli* strains. Such assays are usually aimed at detecting

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TABLE 1. Characteristics of reference strains used in this study

Strain	Serotype	Stx gene(s)[a]	EHEC genotype	Reference(s) or source
H19	O26:H11	stx_1 (*SLT-I*; VT1)		3
933W	O157:H7	stx_2 (*SLT-II*; VT2)		16
87-1215	O157:H7	stx_1, stx_2		This study
B2F1	O91:H21	stx_{2c} (*SLT-IIvha*, *SLT-IIvhb*; VT2c)		15
86-704	O15:H27	stx_{2c} (*SLT-IIvhb*; VT2c)		This study
E32511	O157:NM	stx_2, stx_{2c} (*SLT-II*, *SLT-IIc*)		32
91-126	O128:H?	stx_1 + stx_{2d} (Stx1 + Stx2d)		31; this study
412	O139:K82	stx_{2e} (*SLT-IIe*; VTe)		11, 40
H.I.8	O128:B12	stx_{2f} (*SLT-IIva*; VTeV)		7
90-2380	O157:H7	stx_2	*hlyA eaeA*	This study
25922	O6:H1	Lacking		American Type Culture Collection
92-3136	O157:H21	Lacking		This study
91-0575	O55:H7	Lacking		This study

[a] Data in parentheses are Shiga-like toxin genes, Stx toxins, and/or verotoxins.

the genes stx_1; stx_2; stx_{2e}; $rfbE_{O157}$, which encodes the *E. coli* somatic antigen O157; $fliC_{H7}$, which encodes the *E. coli* structural flagella antigen H7; *uidA*, which encodes β-glucuronidase; EHEC *hlyA* and *eaeA*; and the genes for the cytotoxic necrotizing factors, heat-labile toxin; heat-stable toxin; enteroinvasive toxin, and the enteroaggregative protein (4, 6, 8, 9, 14, 24, 26, 39).

In this study, a multiplex-PCR assay is described that uses three primer sets to detect the genes for 10 *E. coli* O157:H7 genes simultaneously. The genes detected were the eight virulence genes stx_1, stx_2, stx_{2c}, stx_{2d}, stx_{2e}, stx_{2f}, EHEC *hlyA*, and *eaeA* and the two genes $rfbE_{O157}$ and $fliC_{H7}$ to provide genotypic identification of the O157:H7 serotype most commonly associated with disease. As an internal positive control for each reaction, primers were also designated to amplify the *E. coli* 16S rRNA. Validation of the multiplex-PCR primers was performed and interpreted using individual primers by PCR-RFLP analysis (31, 38). In addition, a total of 129 isolates of *E. coli* O157 and non-O157 were characterized for the presence of the various virulence genes.

MATERIALS AND METHODS

Bacterial strains and culture media. A total of 129 *E. coli* isolates derived from the culture collection of the National Laboratory for Enteric Pathogens were used in this study, and these included 79 *E. coli* O157:H7 isolates, five O157:NM (nonmotile) isolates, seven O157:non-H7 isolates (one each of O157:H10, H19, H21, H43, and H45 and two of H16), 12 non-O157:H7 isolates (two isolates of O27:H7, three of O18:H7, five of O55:H7, and one each of O156:H7 and O83:H7), six non-O157:NM isolates (one each of O1:NM, O7:NM, O91:NM, and O rough:NM and two isolates of O111:NM), 14 non-O157:non-H7 isolates (one isolate of O6:H1; two of O103:H2; one each of O146:H21, O26:H11, O70:H11, O91:H21, O139:K82, O128:B12, and O15:H27; two of O128:H untypeable, 1 of O113:H21, and 1 of O rough:H21), three isolates of O untypeable:H7, 1 of O untypable:H8, and 2 of O untypeable:H untypeable. Of these, 101 strains were STEC and 28 were negative for Stx. The control strains had been previously defined in terms of virulence factors and toxigenicity with respect to the genes stx_1, stx_2, stx_{2c}, stx_2-stx_{2c}, stx_{2d}, stx_{2e}, stx_{2f}, EHEC *hlyA*, and *eaeA* (Table 1).

DNA isolation. Total DNA was isolated from 0.5 ml of brain heart infusion broth culture grown overnight for all the bacterial strains used in the study. The procedure used for DNA isolation was as described previously (38). DNA samples were dissolved in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA at pH 8.0), and the DNA concentration was determined in micrograms per milliliter at an optical density reading of A_{260}. The template DNA concentration used was 2 μg/ml.

Primer design. Oligonucleotides ranging from 19- to 25-mers were selected from the published DNA sequences of *E. coli* using Oligo software (version 3.4). Synthesis of oligonucleotides was carried out at the DNA Core Facility at the National Microbiology Laboratory, Winnipeg, Canada. For multiplex PCR, three primers sets were prepared. Set A was designed to amplify stx_1, stx_2, stx_{2f}, and 16S rRNA; set B was designed to amplify stx_{2c}, stx_{2e}, *eaeA*, and 16S rRNA; and set C was designed to amplify stx_{2d}, EHEC *hlyA*, $rfbE_{O157}$, and $fliC_{H7}$ as well as 16S rRNA. The primer sequences used in the multiplex PCR are outlined in Table 2.

Multiplex-PCR conditions. Three sets of primer mixtures were prepared with slight modification according to the instructions supplied with the AmpliTaq Gold kit (Applied Biosystems, Foster City, Calif). In general, all of the multiplex primer sets contained 200 μM deoxynucleoside triphosphates, 2.5 μl of 10× reaction buffer (100 mM Tris-HCl at pH 8.3, 500 mM KCl), 1.5 mM $MgCl_2$, and a 0.1 μM concentration of the *E. coli* 16S rRNA (E16S) primers. In addition to these, set A contained a 0.5 μM concentration of each of the primers Stx1, Stx2, and Stx2f together with 2.5 U of *Taq* DNA polymerase (AmpliTaq Gold; Applied Biosystems) and 5 ng of template DNA. The volume of this mix was adjusted to 25 μl with sterile water. In addition to the common constituents, the multiplex primer set B included primers at the following concentrations: 1.5 μM Stx2c, 0.4 μM Stx2e, and 0.75 μM EAE. Multiplex primer set C comprised common components plus primers at the following concentrations: 1.5 μM Stx2d, 1.0 μM (each) HlyA and RfbE, and 0.4 μM FliC (Table 2). DNA amplification was carried out in a Perkin-Elmer thermocycler 2400 using an initial denaturation step at 95°C for 8 min, followed by 30 cycles of amplification with denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, ending with a final extension at 72°C for 7 min.

RESULTS

Multiplex PCR for the detection of *E. coli* virulence genes and O157:H7 serotype genes. The reaction conditions for the multiplex-PCR assay were optimized to ensure that all of the target gene sequences were satisfactorily amplified. Initially, equimolar primer concentrations of 0.5 μM each were used in the multiplex PCR, but there was uneven amplification, and some of the products were barely visible even after the reaction was optimized for the cycling conditions. Overcoming this problem required changing the proportions of the various primers in the reaction mixture to give an increase in the concentration of primers for the "weak" loci and a decrease in the primer concentration for the "strong" loci. The final concentration of the primers (0.1 to 1.5 μM) varied considerably among the loci and was established empirically.

The primers were designed to target the coding regions of the genes, and care was taken to avoid areas of homology within the structural genes encoding the Stx2 family of toxins. The primers used in each set had identical annealing temperatures, which reduced the possibility of the occurrence of unwanted bands originating from nonspecific amplification. Fig-

TABLE 2. Primers used in this study

Primer set	Primer	Sequence (5′ to 3′)	Target gene	Location within gene	Size of PCR amplicon (bp)	GenBank accession no.
A	Stx1-a	TCTCAGTGGGCGTTCTTATG	stx_1	777–796	338	M17358
	Stx1-b	TACCCCCTCAACTGCTAATA		1114–1095		
	Stx2f-a	TGTCTTCAGCATCTTATGCAG	stx_{2f}	300–320	150	M29153
	Stx2f-b	CATGATTAATTACTGAAACAGAAAC		449–425		
	Stx2-a	GCGGTTTTATTTGCATTAGC	stx_2	1228–1247	115	X07865
	Stx2-b	TCCCGTCAACCTTCACTGTA		1342–1323		
B	Stx2c-a	GCGGTTTTATTTGCATTAGT	stx_{2c}	1186–1205	124	M59432
	Stx2c-b	AGTACTCTTTTCCGGCCACT		1309–1290		
	Stx2e-a	ATGAAGTGTATATTGTTAAAGTGGA	stx_{2e}	204–228	303	M36727
	Stx2e-b	AGCCACATATAAATTATTTCGT		506–485		
	EAE-a	ATGCTTAGTGCTGGTTTAGG	*eaeA*	132–151	248	Z11541
	EAE-b	GCCTTCATCATTTCGCTTTC		379–360		
C	Stx2d-a	GGTAAAATTGAGTTCTCTAAGTAT	stx_{2d}	1221–1244	175	AF043627
	Stx2d-b	CAGCAAATCCTGAACCTGACG		1395–1375		
	HlyA-a	AGCTGCAAGTGCGGGTCTG	EHEC *hlyA*	867–885	569	X79839
	HlyA-b	TACGGGTTATGCCTGCAAGTTCAC		1435–1412		
	RfbE-a	CTACAGGTGAAGGTGGAATGG	$rfbE_{O157}$	673–693	327	S83460
	RfbE-b	ATTCCTCTCTTTCCTCTGCGG		999–979		
	FliC-a	TACCATCGCAAAAGCAACTCC	$fliC_{H7}$	1068–1088	247	AF228488
	FliC-b	GTCGGCAACGTTAGTGATACC		1314–1294		
All[a]	E16S-a	CCCCCTGGACGAAGACTGAC	*E. coli* 16S rRNA	1682–1701	401	AB035924
	E16S-b	ACCGCTGGCAACAAAGGATA		2082–2063		

[a] Used in all sets as the internal control.

ure 1 shows the presence of the amplified product profiles after agarose gel electrophoresis, when DNA extracted from a reference *E. coli* strain (positive control) was used as the template in the PCR using the multiplex primer sets. The four bands in set A, stx_1, stx_2, stx_{2f}, and 16S rRNA, were amplified consistently even when mixtures of DNA derived from the same strains were tested (Fig. 1A). Similarly, four bands were obtained in set B when a mixture of DNA extracts from the corresponding strains that carried the genes stx_{2c}, stx_{2e}, *eaeA*, and 16S rRNA was tested (Fig. 1B). For set C, which contained primers to amplify the genes stx_{2d}, EHEC *hlyA*, $rfbE_{O157}$, $fliC_{H7}$, and 16S rRNA, a total of five bands was obtained from the positive-control DNA (Fig. 1C). The various control strains corresponded to the predicted sizes (Table 2). As a negative control, all sets were tested with *E. coli* strain ATCC 25922, and only the 16S rRNA band was observed (lanes 12 in Fig. 1A and B; lane 11 in Fig. 1C). Genomic DNA from *Aeromonas hydrophila* and *Campylobacter jejuni* was also tested using these three primer sets, and none gave PCR amplification bands (data not shown).

To substantiate the multiplex-PCR technique, 129 strains of *E. coli* that were tested by multiplex PCR were also screened for the presence of individual toxin genes by using the methods described previously (14, 17, 18, 24, 30). stx_2 subtype, stx_{2c}, and stx_{2d} genes were confirmed by PCR-RFLP analysis (31, 38). The serotype identity of the *E. coli* O157:H7 isolates and the non-O157:H7 strains was determined at the National Laboratory for Enteric Pathogens. Agreement between the toxigenic profile and O157:H7 serotype results and the multiplex-PCR data was observed (Table 3). However, 3 of 11 phenotypically NM strains showed gene-positive results for H7 by the PCR assay, and one of these was the reference stain E32511, previously shown to be genotypically H7. An internal control of *E. coli* 16S rRNA was present in all of the *E. coli* samples, thus confirming the presence and the quality of *E. coli* DNA amplification as well as validating the PCR conditions.

Primary validation of amplicons. The sizes of the amplicons obtained from the multiplex primer sets were identical to those predicted from the design of the primers (Table 2). The amplicons from the control strains were subjected to further confirmation and characterization by digestion with restriction endonucleases with cleavage sites within each of the amplicons. The restriction enzymes used and the predicted product sizes are given in Table 4. Enzyme fragments with the anticipated sizes were obtained in all cases (data not shown).

Analysis of results. Among the 129 strains tested, 101 (78.3%) were positive for Stx toxins. All of O157:H7 strains were *eaeA* and EHEC *hlyA* positive. A total of 96 (74.4%) *E.*

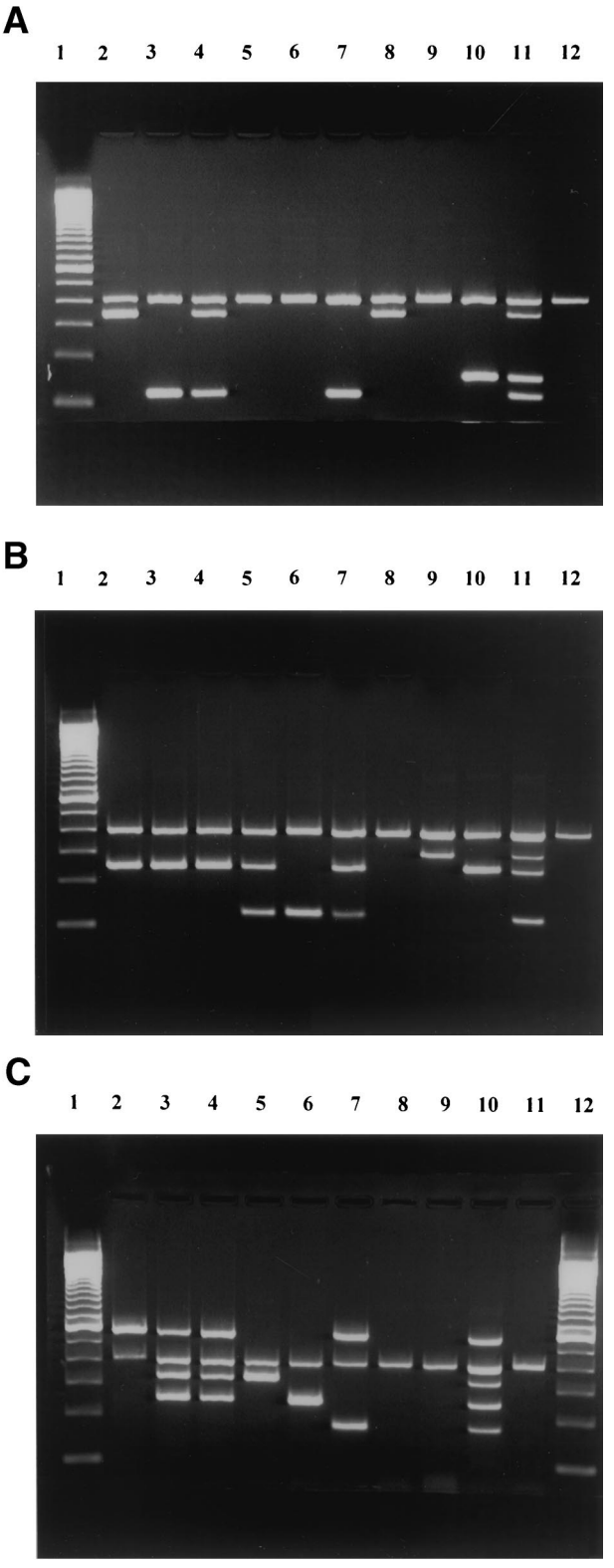

FIG. 1. Shown are multiplex-PCR amplification profiles from reference *E. coli* strains with primer set A (A), B (B), and C (C). (A) Lane 1, 100-bp DNA ladder (Bethesda Research Laboratories Inc., Gaithersburg, Md.); lane 2, stx_1 and 16S rRNA (*E. coli* strain H19); lane 3, stx_2 and 16S rRNA (strain 90-2380); lane 4, stx_1, stx_2, and 16S rRNA (strain 87-1215); lane 5, 16S rRNA (formerly stx_{2Va}, strain 91-2245); lane 6, 16S rRNA (formerly stx_{2Vb}, strain 86-704); lane 7, stx_2 (stx_2-stx_{2c}, strain E32511); lane 8, stx_1 and 16S rRNA (stx_1-stx_{2d}, strain 91-126); lane 9, 16S rRNA (stx_{2e}, strain 412); lane 10, stx_{2f} and 16S rRNA (stx_{2f}, strain H.I.8); lane 11, stx_1, stx_2, stx_{2f}, and 16S rRNA; lane 12, *stx* negative control (ATCC 25922). (B) Lane 1, 100-bp DNA ladder (Bethesda Research Laboratories Inc.); lane 2, amplification products of *eaeA* and 16S rRNA (stx_1, strain H19); lane 3, *eaeA* and 16S rRNA(stx_2, strain 90-2380); lane 4, *eaeA* and 16S rRNA(stx_1-stx_2, strain 87-1215); lane 5, *eaeA*, stx_{2c}, and 16S rRNA (formerly stx_{2Va}, strain 91-2245); lane 6, stx_{2c} and 16S rRNA (formerly stx_{2Vb}, strain 86-704); lane 7, *eaeA*, stx_{2c} and 16S rRNA (stx_2-stx_{2c}, strain E32511); lane 8, 16S rRNA(stx_1-stx_{2d}, strain 91-126); lane 9, stx_{2e} and 16S rRNA (stx_{2e}, strain 412); lane 10, *eaeA* and 16S rRNA (stx_{2f}, strain H.I.8); lane 11, *eaeA*, stx_{2c}, stx_{2e}, and 16S rRNA; lane 12, *stx* negative control (ATCC 25922). (C) Lanes 1 and 12, 100-bp DNA ladder (Bethesda Research Laboratories Inc.); lane 2, *hlyA* and 16S rRNA (strain H19); lane 3, *hlyA*, $rfbE_{O157}$, $fliC_{H7}$, and 16S rRNA(strain 90-2380, O157:H7); lane 4, *hlyA*, $rfbE_{O157}$, $fliC_{H7}$, and 16S rRNA (strain E32511, O157: NM); lane 5, $rfbE_{O157}$ and 16S rRNA (O157: H21); lane 6, $fliC_{H7}$ and 16S rRNA (O55: H7); lane 7, *hlyA*, stx_{2d}, and 16S rRNA (stx_1-stx_{2d}, strain 91-126); lane 8, 16S rRNA (strain 412); lane 9, 16S rRNA (strain H.I.8); lane 10, positive control of *hlyA*, $rfbE_{O157}$, $fliC_{H7}$, stx_{2d}, and 16S rRNA; lane 11, *stx* negative control (ATCC 25922).

coli isolates were positive for the *eaeA* gene, while seven *eaeA* positives were detected among strains otherwise lacking *stx*. The ability of the C set of primers to identify O157:H7 from other *E. coli* strains was determined by analyzing 79 O157:H7, 5 O157:NM, 7 O157:non-H7, and 38 non-O157 *E. coli* isolates. Two of the five O157:NM strains and one of six non-O157:NM strains were $fliC_{H7}$ gene positive, indicating that these isolates were genetically H7 with flagellum antigens that were either not expressed or not detectable in serotyping tests (Table 3) (5). All of the 129 samples tested contained the *E. coli* 16S rRNA gene.

DISCUSSION

STEC strains have been associated with outbreaks of disease that included cases of HC and HUS in humans. The two major categories of *E. coli* Stx toxins are Stx1 and Stx2. Stx1 is a relatively homogeneous family of toxins that show identity with the Shiga toxins of *Shigella dysenteriae*. Stx2 toxins, however, are a more heterogeneous group that are serologically distinct from Stx1. Within the Stx2 toxin family, Stx2c was formerly subdivided into Stx2-Va and Stx2-Vb (15, 38). These are only partially neutralized by antiserum to Stx2 (14; Head et al., letter). Stx2d shows a low cytotoxicity in Vero cells (27, 28, 31), while Stx2e is cytotoxic only in Vero cells and has been associated with porcine edema disease (11, 21). Stx2f (also called VTeV) has low-level cytotoxicity for Vero cells and is readily neutralized by antisera that are raised against Stx2 and Stx2e (7, 35).

Among STEC isolates, certain strains appear to have a greater degree of virulence for humans, while some data suggest that toxin type is important in determining the probability of developing HUS. Indeed, it has been shown epidemiologically that Stx2 is more critical than Stx1 for the development of HUS, in that strains producing Stx2 were more frequently associated with cases of HUS than were those isolates expressing Stx1 only (10). In addition to serological differences, the Stx2 group of toxins may differ in terms of their in vitro or in vivo properties. Experiments with clones carrying chimeric

TABLE 3. Major virulence genes detected by the three primer sets in the multiplex PCR analysis

Toxin(s) confirmed by PCR or PCR-RFLP analysis (*n*)[a]	Serotype (*n*)[b]	No. of genes detected by PCR with primer set:									
		A			B			C			
		stx_1	stx_2	stx_{2f}	stx_{2c}	stx_{2e}	*eaeA*	stx_{2d}	*hlyA*	$rfbE_{O157}$	$fliC_{H7}$
Stx1 (13)	Non-O157:non-H7 (5)	5	—[c]	—	—	—	4	—	5	—	—
	O111:NM (1)	1	—	—	—	—	1	—	1	—	—
	O91:NM (1)	1	—	—	—	—	—	—	1	—	—
	O157:H7 (5)	5	—	—	—	—	5	—	5	5	5
	O157:NM (1)	1	—	—	—	—	1	—	1	1	1
Stx1 + Stx2 (27)	O UT:H UT[d] (1)	1	1	—	—	—	—	—	—	—	—
	O157:H7 (26)	26	26	—	—	—	26	—	26	26	26
Stx1 + Stx2c (2)	O157:H7 (1)	1	—	—	1	—	1	—	1	1	1
	O111:NM (1)	1	—	—	1	—	1	—	—	—	—
Stx1 + Stx2d (3)	O128:H? (2)	2	—	—	—	—	—	2	2	—	—
	O rough:NM (1)	1	—	—	—	—	—	1	—	—	—
Stx2 (16)	O157: H7 (16)	—	16	—	—	—	16	—	16	16	16
Stx2 + Stx2c (27)	O157:NM (1)	—	1	—	1	—	1	—	1	1	1
	O157:H7 (26)	—	26	—	26	—	26	—	26	26	26
Stx2c (10)	O91:H21 (1)	—	—	—	1	—	—	—	1	—	—
	Non-O157:non-H7 (3)	—	—	—	3	—	—	—	1	—	—
	O157:H7 (5)	—	—	—	5	—	5	—	5	5	5
	O55:H7 (1)	—	—	—	1	—	1	—	—	—	1
Stx2d (1)	O UT:H8 (1)	—	—	—	—	—	—	1	—	—	—
Stx2e (1)	O139:K82 (1)	—	—	—	—	1	—	—	—	—	—
Stx2f (1)	O128:B12 (1)	—	—	1	—	—	1	—	—	—	—
Stx negative (28)	O157:non-H7 (7)	—	—	—	—	—	6	—	—	7	—
	O157:NM (3)	—	—	—	—	—	—	—	—	3	—
	Non-O157:H7 (11)	—	—	—	—	—	1	—	—	—	11
	O UT:H7 (3)	—	—	—	—	—	—	—	—	—	3
	Non-O157:NM (2)	—	—	—	—	—	—	—	—	—	1
	Non-O157:non-H7 (1)	—	—	—	—	—	—	—	—	—	—
	O UT:H UT (1)	—	—	—	—	—	—	—	—	—	—
Total	101/129	45	70	1	39	1	96	4	92	91	106

[a] As outlined in references 17, 18, 31, and 38.
[b] Numbers in parentheses are numbers of serotypes tested.
[c] —, tested negative for the gene.
[d] UT = untypable.

O48/OX3b stx_2 operons indicated that the increased virulence was a function of the A subunit of $stx_{2/OX3b}$. This differs in the A-subunit structure from that of $stx_{2/O48}$ by only two amino acids (Met-4→Thr and Gly-102→Asp, respectively). These findings raise the possibility that naturally occurring Stx2 sequence variations may directly impact the capacity of a given Stx-producing *E. coli* strain to cause severe disease (29).

The use of multiplex PCR or PCR-RFLP analysis to characterize the various subtypes of the stx_2 genes has been well documented (4, 6, 8, 9, 14, 24–26, 31, 38). Lin et al. (19) introduced common primers for PCR-RFLP analysis in order to detect the genes for various Stx toxins. However, all the PCR-related methods require restriction digestion to achieve identification. A multiplex PCR-based diagnostic protocol is described for the detection of those genes encoding the various

TABLE 4. Predicted sizes of restriction fragments and enzymes used for RFLP analysis of amplified products of multiplex PCR

Genes	PCR amplicon size (bp)	Multiplex primer set(s)	Enzyme	Expected sizes of restriction fragments (bp)
stx_1	338	A	*Bgl*I	136, 202
stx_2	115	A	*Bsr*DI	37, 78
stx_{2f}	150	A	*Alu*I	54, 96
stx_{2e}	303	B	*Taq*I	51, 87, 165
stx_{2c}	124	B	*Hha*I	48, 76
eaeA	248	B	*Alu*I	109, 139
stx_{2d}	175	C	*Rsa*I	66, 109
EHEC *hlyA*	569	C	*Apa*I	299, 270
$rfbE_{O157}$	327	C	*Alu*I	80, 93, 154
$fliC_{H7}$	247	C	*Alu*I	40, 207
E. coli 16S rRNA	401	A, B, and C	*Rsa*I	156, 245

Stx toxins, including Stx1, Stx2, Stx2c, Stx2d, Stx2e, and Stx2f, EHEC *hlyA*, and *eaeA*, together with the genes governing the serotype O157 (*rfbE*) and H7 (*fliC*) in the absence of restriction enzyme digestion. Compared to the individual primers and PCR-RFLP analysis results, the multiplex-PCR primer sets proved to be highly specific, they gave consistent results, and they were effective in detecting all 11 genes, including the internal control gene. All primers were gene specific, as demonstrated by restriction fragment lengths obtained after specific restriction endonuclease digestion of the amplicons.

In this study, the toxin genotype and O157:H7 serotype of a range of *E. coli* strains was demonstrated (Table 3). Of 81 STEC O157:H7 clinical isolates (including the 2 that were serotypically O157:NM but were PCR positive for $fliC_{H7}$), all were positive for the EHEC *hlyA* and *eaeA* genes. These finding were in agreement with previous reports (1, 33). Interestingly, among 20 of the non-O157 STEC isolates, 11 (55%) were EHEC *hlyA* positive and 8 (40%) were *eaeA* positive. Three of 10 strains positive for the stx_{2c} gene only were negative for EHEC *hlyA*, while the reference stx_{2c} gene-positive strain (serotype O91:H21, an isolate from a patient with HUS) was EHEC *hlyA* positive and lacked *eaeA*, suggesting that the *eaeA* may not be an essential major virulence factor for the acquisition of HUS. All four strains that possessed the stx_{2d} genotype lacked *eaeA*. Of these four strains, three possessed both the stx_1 and stx_{2d} genotype, while one was positive for stx_{2d} alone. Two of these were *hlyA* positive. This suggested that STEC strains without EHEC *hlyA* may possess reduced pathogenicity or may even be nonpathogenic in humans (36). Furthermore, among the 28 non-STEC isolates (Stx negative), 7 were *eaeA* positive and none possessed the EHEC *hlyA* gene. This suggested that EHEC *hlyA* may be a more critical virulence factor for disease than *eaeA* (Table 3). Further studies are required on EHEC *hlyA*, *eaeA*, and the various Stx components in order to elucidate the role that these toxins play in STEC disease in general and in HUS and HC in particular.

In total 11 phenotypic nonmotile *E. coli* isolates were analyzed. Of these, three were $fliC_{H7}$ positive. Two of these were O157:NM strains (including reference strain E32511), and one was an O1:NM strain. All three were confirmed as H7 positive using primer $FLIC_{h7}$-F and $FLIC_{h7}$-R (14). An *E. coli* $fliC_{H7}$ sequence comparison (GenBank accession no. AF228487 for O157:H7, AF228495 for O19ab:H7, AF228496 for O53:H7, AF228489 for O55:H7, and U47614 for O157:NM) also confirmed that *fliC* is highly conserved among different serogroups. Therefore, it would appear that some of *E. coli* strains that are serologically NM are genetically H7 (5). The factors that govern *fliC* expression are unclear; however, environmental factors or other related genes may play key roles.

From these data we conclude that the multiplex primer sets described are specific and give highly consistent results. The use of this three-tube assay should allow simultaneous detection of the major virulence factors associated with *E. coli* O157 and non-O157 strains and avoids the need for endonuclease digestion steps.

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