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Direct Detection of Verotoxin-Producing *Escherichia coli* in Stool Samples by PCR

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A method for the rapid detection of verotoxin-producing *Escherichia coli* in stool samples by PCR was evaluated. Verotoxin-1 and verotoxin-2 genes in DNA extracted directly from stool samples were amplified with oligonucleotide primers. Stools spiked with control organisms, *E. coli* C600 (H19B) (verotoxin-1) or *E. coli* C600 (933W) (verotoxin-2), demonstrated that verotoxin-1-containing organisms could be detected at 10^2 CFU per 0.1 g of stool and verotoxin-2-containing organisms could be detected at 10^7 CFU per 0.1 g of stool. Testing of stool samples from patients with diarrhea showed a high concordance between PCR positivity and the presence of verotoxin-producing *E. coli*, determined by isolation of serotype O157:H7 on sorbitol-MacConkey medium (34 of 35 stool samples) or by colony blots with gene probes (19 of 21 stool samples). Conversely, only 1 of 20 (5.0%) stool samples that were O157:H7 culture negative and colony blot negative and that contained free verotoxin only was positive by PCR. As well, only 4 of 145 (2.8%) stool samples that were negative for serotype O157:H7 or free verotoxin were PCR positive. PCR of DNA extracted directly from stool samples provides a rapid method for the detection of stool samples containing verotoxin-producing *E. coli* compared with colony blot testing.

Verotoxin-producing *Escherichia coli* (VTEC) is an important cause of hemorrhagic colitis, hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura in humans (23). Strains of VTEC associated with bloody diarrhea and HUS are also referred to as enterohemorrhagic *E. coli*. This class of diarrheagenic *E. coli* produces several verocytotoxins (verotoxins; VTs) and carries the genetic determinants for attaching-and-effacing lesions to the bowel mucosa (16, 23, 40). Clinical isolates of VTEC produce two types of VTs, VT-1 and several variants of VT-2. VT-1 and VT-2 are immunologically distinct and demonstrate approximately 58% gene sequence homology (23).

Epidemiologic and microbiologic studies have demonstrated that *E. coli* serotype O157:H7 accounts for 40 to 70% of VTEC infections (16, 23). In routine clinical practice, *E. coli* O157:H7 is recovered from stool samples on sorbitol-containing MacConkey medium (SMAC) because this serotype does not ferment sorbitol rapidly (16). Other VTEC serotypes are not routinely identified.

Additional nonroutine methods for the diagnosis of VTEC infections include (i) testing of stool samples for free VT (FVT) using Vero cell monolayers and specific neutralizing anti-VT antibodies (24, 36), (ii) enzyme-linked immunosorbent assays for toxins in culture supernatants or fecal filtrates (3, 9), (iii) colony hybridizations with VT gene probes (6, 34), and (iv) PCR amplification of VT gene segments in VTEC isolates (4, 5, 12, 19, 22, 29–31).

PCR methods have been developed to detect VT gene sequences in *E. coli* recovered from clinical samples (4, 19, 22, 30, 31) and ground beef (12). PCR-based methods for the direct detection of VTEC in feces (5) or fecal broth cultures (29) have recently been reported. However, these studies did not compare PCR results with those of other assay methods and did not examine large numbers of stool samples.

Here we report the usefulness of PCR as a means of detecting VT genes in DNA extracted directly from stool samples from patients with diarrhea and compare these results with those of colony blot hybridization assays, assays for FVT, and cultures for *E. coli* O157:H7.

(Preliminary results of this study have been presented at the 33rd Interscience Conference on Antimicrobial Agents and Chemotherapy, New Orleans, La., 17 to 20 October 1993 [25a], and at the 62nd Annual Meeting of the Royal College of Physicians and Surgeons of Canada, Vancouver, British Columbia, Canada, September 1993 [abstr. 515].)

MATERIALS AND METHODS

Stool samples, cultures for *E. coli* O157:H7, and testing for FVT in fecal filtrates. Samples were collected during the summer months from patients presenting at the outpatient clinics of the Calgary General Hospital (CGH; July to September 1990, April to September 1993), the Alberta Children's Hospital (ACH; May to August 1992), and the Calgary Medical Laboratories (CML; July to September 1990). Stool samples were immediately cultured for enteric pathogens by standard methods (2) and for *E. coli* O157:H7 on SMAC medium (25) and were examined for ova and parasites (13). Stool samples from CGH and CML were stored in 10% glycerol–brain heart infusion (BHI) broth at −70°C. Stool samples from ACH were stored without preservatives at −70°C. Stool samples collected at CGH (n = 281) and CML (n = 3,296) in the summer of 1990 were part of a community prevalence study for VTEC. These stool samples were all cultured for *E. coli* O157:H7 and tests for FVT were performed on stool filtrates by using Vero cell monolayers (24) and then monoclonal antibody neutralization.

Stool filtrates were made by mixing 1 g of the stool sample with 1 ml of phosphate-buffered saline (PBS) and were tested undiluted for their cytotoxic activities on Vero cell monolayers. Cytotoxic filtrates were retested and confirmed for VT-1 or VT-2 by using specific monoclonal antibodies against the respective toxins. Stool filtrates were serially diluted twofold and were mixed with either sterile PBS, 10 U of monoclonal antibody 13C4 (specific for VT-1), 10 U of BC5 BB12 (specific for VT-2), or a combination of both (8, 36). One unit of antibody is the amount necessary to neutralize 1 50% cytotoxic dose of homologous toxin. The preparations were incubated at 37°C for 1 h and were then

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layered onto Vero cell monolayers and incubated for 72 h at 37°C in 5% CO_2. *E. coli* C600 (H19B) and *E. coli* C600 (933W) were used as positive controls for VT-1 and VT-2, respectively. *E. coli* ATCC 25922 was used as a negative control. Complete neutralization was defined as the absence of cytotoxic activity. Significant neutralization was defined as a fourfold reduction in titer.

Colony blot probes and PCR of isolates. Any sample that was positive for serotype O157:H7 or FVT was probed by a colony blot assay with the VT-1 and VT-2 gene probes of Newland and Neill (27). One gram of stool was serially diluted 10-fold from 10^{-2} to 10^{-8} in PBS and was spread onto MacConkey agar, and the plates were incubated overnight at 37°C. Fifty lactose-positive colonies were picked and subcultured onto fresh plates, and the plates were incubated for 8 h at 37°C. These colonies were lifted onto nylon membranes (Magnagraph Nylon; 0.45-μm pore size; MSI Separations Inc., Westboro, Mass.) and were probed as described elsewhere (17). A single probe-positive colony was arbitrarily chosen from each sample as the representative VTEC strain. Colony blot-positive isolates were retested for the VT-1 and VT-2 genes by Southern hybridization with gene probes (27) or by PCR with the primers of Pollard et al. (30) (all isolates) and additional primers of Gannon et al. (12) and Karch and Meyer (22) for non-O157:H7 strains. Stool samples collected from ACH in 1992 and CGH in 1993 were cultured only for *E. coli* O157:H7.

Serotyping and serogrouping. Sorbitol-negative *E. coli* isolates recovered on SMAC plates were O (*E. coli* O157 Latex Test; Oxoid, Hampshire, United Kingdom) and H (Difco, Detroit, Mich.) typed with commercial sera. *E. coli* isolates that were positive for the VT-1 or VT-2 gene, or both, by colony blot assays and which produced VT in vitro were serotyped by H. Lior of the National Laboratory for Enteric Pathogens, Laboratory Centre for Disease Control in Ottawa, Ontario, Canada, or R. Wilson at the Pennsylvania State University *E. coli* Reference Center (the remaining strains).

In vitro toxin production. All colony blot-positive *E. coli* strains were tested for in vitro toxin production by inoculating a representative colony into 5 ml of BHI broth (Difco), syncase broth (Difco) without and with iron depletion with Chelex 100 (Bio-Rad Laboratories, Richmond, Calif.) (30), and Luria-Bertani medium (Difco) supplemented with 0.2 mM 2,2′-dipyridyl (7) (Sigma Chemical Co.). Broths were incubated overnight at 37°C in room air in a shaking incubator at 300 rpm. The supernatant was passed through a 0.45-μm-pore-size filter. If the supernatant did not show the presence of VT, the bacterial pellet was treated with polymyxin B (32) and the resulting supernatant was retested for toxin activity. If the initial test was negative, isolates were tested three more times for VT production in vitro.

PCR primers. The primer pairs selected to amplify VT-1 and VT-2 gene segments (5′-3′) (VT-1, GAAGAGTCCGTGGGATTACG and AGCGATG CAGCTATTAATAA; VT-2, TTAACCACACCCACGGCAGT and GCTCTG GATGCATCTCTGGT) have been used previously for PCR of VTEC (30). The VT-1 primers amplify a 130-bp fragment (nucleotides 1191 to 1320 of the VT-1 gene), and the VT-2 primers amplify a 346-bp fragment (nucleotides 426 to 771 of the VT-2 gene).

Primers were synthesized by the Regional DNA Synthesis Laboratory, University of Calgary, Calgary, Alberta, Canada, and were gel purified before use.

Processing of samples for PCR. Stool samples (0.1 g) were mixed with 0.9 ml of PBS and were centrifuged at 2,000 rpm (Eppendorf 5415C microcentrifuge) for 2 min to remove the debris. The supernatant was transferred to a new tube and the tube was centrifuged at 12,000 rpm (Eppendorf 5415C microcentrifuge) for 5 min. The pellet was washed twice with 1.0 ml of PBS and was resuspended in 0.5 ml water–0.01 ml of 10% sodium dodecyl sulfate (SDS). This cell suspension was boiled in a water bath for 10 min, cooled, and then extracted twice with phenol-chloroform. The upper aqueous layer was recovered, and the DNA was precipitated in isopropanol and resuspended in 0.03 ml of water. One microliter of this DNA was used in the PCR.

PCR protocol. The PCR mixture consisted of 5 μl of 10× PCR buffer (final concentration, 50 mM KCl, 0.01% gelatin, 10 mM Tris-HCl [pH 8.3]), 2.5 mM $MgCl_2$, 0.2 mM (each) dATP, dCTP, dGTP, and dTTP, 0.001 mM (each) VT-specific primer pair, and 1 μl of DNA in a final volume of 50 μl. PCR assays were set up as an individual primer set (i.e., DNA was amplified by VT-1 or VT-2 primers in separate tests). The PCR was started with 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus), and the PCR mixture was overlaid with mineral oil and processed in a DNA thermal cycler (Perkin-Elmer Cetus). The cycling conditions consisted of initial denaturation at 95°C for 5 min and then 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. In the final cycle, extension was carried out at 72°C for 7 min. A reagent blank (containing all the components of the reaction mixture with water instead of template DNA) and a VT-negative stool sample spiked with *E. coli* C600 (H19B) (VT-1) or *E. coli* C600 (933W) (VT-2) were run as controls in every PCR procedure. For the final quarter of the study samples, *E. coli* O157:H7 ATCC 43890 (VT-1) and ATCC 43899 (VT-2) were used as additional controls. Stool samples showing positive results by PCR were retested on two further occasions several days and 4 to 8 weeks later to examine the reproducibility of PCR testing.

Amplified products from the PCRs were electrophoresed on 2% agarose gels and were stained with ethidium bromide. A 1-kb molecular size ladder (Gibco BRL, Gaithersburg, Md.) was run with each gel.

Confirmation of PCR amplification products. Amplified products from PCR assays for a representative number of stool samples as well as stool samples spiked with *E. coli* C600 (H19B) or *E. coli* C600 (933W) were confirmed as VT gene products by Southern hybridizations. Briefly, amplified products electrophoresed on agarose gels were transferred onto nylon membranes and were probed with either a 614-bp VT-1 or a 779-bp VT-2 gene probe. The DNA probes were synthesized by PCR amplification of purified VTEC genomic DNA with the primers specified by Gannon et al. (12), purified, and labelled with digoxigenin (DIG)-dUTP (DIG-dUTP:dTTP ratio, 1:19) (Boehringer Mannheim Canada Ltd., Laval, Quebec, Canada). Southern blots were developed with reagents from the DIG DNA labelling and detection kit (Boehringer Mannheim) according to the manufacturer's instructions.

Specificity of PCR. VT-1 and VT-2 primer specificities were tested with the following gram-negative organisms in mixed cultures: (i) known VT-negative isolates *Shigella sonnei* ATCC 25931, *Salmonella typhimurium* ATCC 14028, and *Klebsiella oxytoca* (clinical isolate) and (ii) known VT-positive isolates *E. coli* C600 (H19B) (VT-1) and *E. coli* C600 (933W) (VT-2). Organisms were grown overnight as pure cultures to the stationary phase in BHI broth at 37°C. Mixed cultures were made by adding 0.1 ml each of *S. sonnei* ATCC 25931, *S. typhimurium* ATCC 14028, and *K. oxytoca* alone or in combination with each of the VT gene-positive control strains. Culture suspensions were boiled for 10 min, and 10 μl was subjected to PCR analysis.

DNAs extracted from five stool samples from healthy volunteers and 10 stool samples culture negative for enteric pathogens from patients hospitalized for nondiarrheal illnesses were also tested for VT-1 and VT-2 genes.

Sensitivity of PCR assay. A single colony of each of *E. coli* C600 (H19B) and *E. coli* C600 (H933W) and two additional clinical strains each of VT-1- and VT-2-producing *E. coli* were incubated in 5 ml of BHI broth for 4 h in a shaking water bath at 37°C and were adjusted to 0.5 McFarland units (10^8 CFU/ml). Serial 10-fold dilutions of this culture were made in PBS and were used to spike 0.1 g of stool to yield concentrations of 10^1 to 10^7 CFU per 0.1 g of stool. DNA was isolated from the samples and was amplified for VT-1 and VT-2.

RESULTS

PCR amplification of DNA from control (spiked) and patient stool samples. DNA isolated from stool samples spiked with indicator organisms [*E. coli* C600 (H19B) or *E. coli* C600 (933W)] or patient stool samples were amplified as described earlier. Discrete bands corresponding to the expected VT-1 or VT-2 amplicons were seen on the agarose gels (Fig. 1). Southern hybridizations with VT gene probes confirmed that these amplicons were VT gene products (Fig. 1).

Primer specificity for VTEC. Mixed cultures containing *S. sonnei* ATCC 25931, *S. typhimurium* ATCC 14028, and *K. oxytoca* were negative for both VT-1 and VT-2. Mixtures containing these three organisms and *E. coli* C600 (H19B) or *E. coli* C600 (933W) were positive for VT-1 or VT-2, respectively.

All 5 stool samples from healthy volunteers and the 10 stool samples from hospitalized patients were negative for both VT-1 and VT-2.

PCR sensitivity. Five different stool samples spiked with *E. coli* C600 (H19B) or *E. coli* C600 (933W) in the range of 10^1 to 10^7 CFU per 0.1 g of stool were tested by PCR. VT-1-containing *E. coli* C600 (H19B) could be detected in spiked stool samples at 10^2 and 10^3 CFU per 0.1 g in four of five samples and at 10^4 CFU per 0.1 g in all five samples. Stool samples spiked with 10 CFU per 0.1 g or to which indicator strains were not added were all negative.

Sensitivity experiments repeated with stool samples spiked with VT-2-containing *E. coli* C600 (933W) showed that the limit of detection was 10^7 CFU per 0.1 g of stool. Stools spiked with a clinical isolate of *E. coli* O157:H7 positive for both VT-1 and VT-2 showed similar thresholds of detection of VT-1 and VT-2 compared with the results obtained with the *E. coli* C600 lysogens. The clinical strain was tested five additional times, with similar results.

PCR of stools which were cultured for O157:H7, colony blot probed for VTEC, and tested for FVT. Of the 3,577 stool samples submitted for culture for enteric pathogens, 76 (2.1%) were positive for markers of VTEC infection. Categories of infection included FVT positivity and isolation of *E. coli* O157:H7 ($n = 20$), *E. coli* O157:H7 isolation with negative results on FVT testing ($n = 2$), and FVT detected with and without positive colony blot results ($n = 54$). Sufficient stool

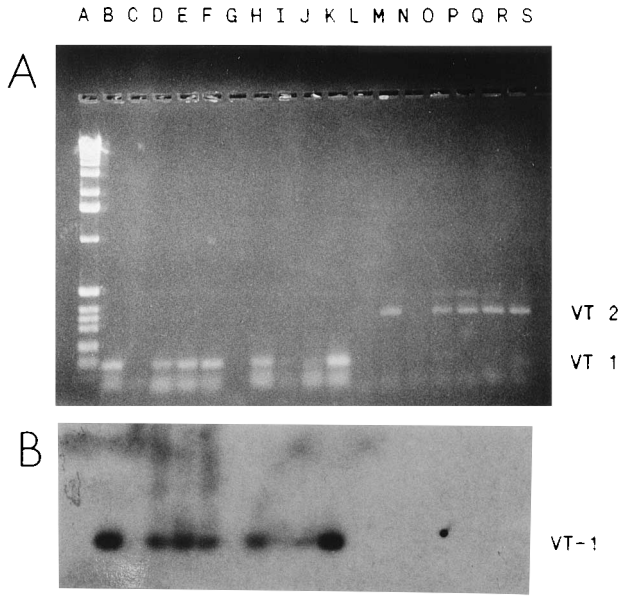

FIG. 1. PCR products obtained from amplification of DNA from control (spiked) and patient stool samples. (A) Lane A, molecular mass markers; lanes B to K, samples amplified with VT-1 primers; lanes B and K, stool spiked with 10^7 CFU of *E. coli* C600 (H19B) per 0.1 g; lane C, stool spiked with *E. coli* C600 (933W); lanes D, E, F, and H, stools that were PCR, colony blot, and FVT positive; lane G, stool that was PCR negative but colony blot and FVT positive; lane I, stool that was PCR and colony blot negative but FVT positive; lane J, stool that was PCR, colony blot, and FVT negative; lanes L to S, samples amplified with VT-2 primers; lane L, stool spiked with 10^6 CFU of *E. coli* C600 (933W) per 0.1 g; lane M, stool spiked with 10^7 CFU of *E. coli* C600 (933W) per 0.1 g; lane N, stool spiked with 10^7 CFU of *E. coli* C600 (H19B) per 0.1 g; lanes O to R, stools that were PCR, colony blot, and FVT positive; lane S, stool that was PCR and colony blot negative but FVT positive. (B) Southern hybridization analysis of PCR products from the gel shown in panel A. PCR products were probed with a DIG-UTP-labelled 614-bp (VT-1) probe.

samples were available from 56 of 76 specimens for the present study. Therefore, these stool samples were VTEC positive by any combination of isolation of serotype O157:H7, positive colony blots, or FVT.

E. coli O157:H7 was recovered from 15 of 56 samples, all of which were PCR positive (Table 1). As well, 19 of 21 stool samples that were *E. coli* O157:H7 culture negative, but colony blot and FVT positive, were also PCR positive (concordance = 0.950). Twenty of the 56 stool samples were positive for FVT only, and 1 of these was PCR positive (concordance = 0.05; Table 1). No enteric pathogen was recovered from this sample.

TABLE 1. Detection of VT genes in stool samples from patients with diarrhea

Stool type	No. of stool samples		Concordance
	Total	PCR positive	
1. Stools tested for O157:H7, FVT, and CBs[a]			
O157:H7, CB, FVT positive	14	14	1
O157:H7, CB positive	1	1	1
CB, FVT positive	21	19	0.950
FVT positive	20	1	0.050
2. Stools tested for O157:H7 and FVT, O157:H7/FVT negative	44	1	0.023
3. Stools tested for O157:H7 only			
O157:H7 positive	20	19	0.950
O157:H7 negative	101	3	0.030

[a] CB, colony blots.

PCR of VTEC-negative stools. The 44 VTEC-negative samples represent a randomly selected subset of the 3,577 samples described above. They were culture negative for enteric pathogens and were FVT negative. Only 1 of 44 (2.3%) was positive by PCR (Table 1).

PCR of stools cultured for *E. coli* O157:H7. One hundred fourteen stool samples were collected from ACH during the summer of 1992 and were stored at −70°C without glycerol preservation. PCR testing was done 9 months later. At that time these samples were replated onto sorbitol-MacConkey medium, and only one contained viable gram-negative organisms. On initial culture, 13 of these samples had been *E. coli* O157:H7 positive, and 12 of these were PCR positive. Three of the remaining 101 stool samples that were *E. coli* O157:H7 negative were PCR positive (Table 1). No enteric pathogens were identified in these three stool samples. Thus, PCR was able to identify three additional stool samples as presumptively VTEC positive that were negative on initial cultures.

Seven O157:H7-positive stools were collected from the CGH microbiology laboratory during the summer of 1993. PCR was done at the time of collection and all were positive. In total, 19 of 20 stool samples that were initially positive for serotype O157:H7 were positive by PCR.

Overall, 34 of 35 (97.1%) stool samples positive for O157:H7 were PCR positive. Similarly, 19 of 21 (90.5%) stools that were O157:H7 negative and colony blot positive were PCR positive. However, only 1 of 20 (5.0%) FVT-positive stools and 4 of 145 (2.8%) O157:H7-negative stool samples were PCR positive. Repeated testing of PCR-positive stool samples showed identical results.

Table 2 provides the correlation of results of cultures of stool samples for *E. coli* O157:H7 on SMAC plates, direct PCR of stool samples, colony blot testing of stool samples, PCR of the isolates recovered from the stool samples, in vitro toxin production by the isolates recovered from stool samples, and FVT type in fecal filtrates. Of the 36 stool samples which were colony blot positive with the Newland and Neill (27) probes, 34 (94.4%) were positive by PCR. However, complete concordance of results by VT type was shown in only 4 of 36 samples when all five types of tests were considered. On the basis of the premise that gene-based diagnostics should yield similar results, a concordance analysis of direct PCR of stool samples, colony blot isolate toxin type, and PCR of that isolate for VT-1 and VT-2 genes showed that 26 of 36 (72%) showed identical results. Similarly, identical results were found in 28 of 36 (78%) samples when direct PCR of stool samples and colony blots were compared and in 27 of 36 (75%) samples when direct PCR of stool samples and PCR of the isolate were compared. When toxin types in stool samples determined by PCR were compared with the FVT types, agreement was observed for only 17 of 36 samples. For organisms which produced toxin in vitro, a comparison of in vitro and in vivo toxin production as exemplified by FVT results showed concordance for 17 of 24 (71%) samples.

Although all of the isolates producing VT in vitro were PCR positive with the primer sets of Pollard et al. (30) and also reacted positively to the primers of Karch and Meyer (22), the 11 strains which did not produce toxin in vitro with and without polymyxin B extraction of the bacterial pellet were less predictable. Eight of 11 strains were positive with the primers of Pollard et al. (30), whereas all 11 strains were positive with the

TABLE 2. Correlation of stool cultures, PCR of stools, colony blots of stools, PCR of stool isolates with the primers of Pollard et al. (30), in vitro toxin production by stool isolates, and FVT in fecal filtrates

No. of stool samples with listed results	Recovery of *E. coli* O157:H7 on SMAC plates	Result of PCR of stool	Result of colony blot of stools	Colony blot isolate serotype or group	PCR of stool isolate with primers of Pollard et al. (30)	In vitro toxin production by stool isolates	FVT in fecal filtrates
2	+	1,2	1,2	O157:H7	1,2	1,2	1,2
5	+	1,2	1,2	O157:H7	1,2	2	1,2
4	+	1,2	1,2	O157:H7	1,2	2	2
1	+	1,2	1,2	O157:H7	1	2	2
1	+	1,2	2	O157:H7	2	2	2
1	+	1	1,2	O157:H7	1	1,2	1,2
1	+	1	2	O75:NM	2	Negative	Negative
2	−	1,2	1,2	O157:H7	1,2	2	2
1	−	1,2	1,2	OR:H	1,2	2	1,2
2	−	1	1	O103:H25(2)	1	1	1
1	−	Negative	1	O103:H2	1	1	1
1	−	Negative	1	O153:H11	1	1	1
1	−	1	1	O126:H11	1,2	1	1
1	−	1	1,2	O126:H11	1,2	1,2	1
1	−	1,2	1,2	O2:H29	1,2	1,2	1,2
3	−	1	1	O17:U;O2:U;O2:14,32	1(1)[a]	Negative	1
1	−	1	1	—:48	Negative (1)	Negative	1,2
2	−	1	1	O19,O133:NM;O2:4,32,38	1(1)	Negative	1
3	−	1	1	O142,X13:U;O26:NM;OX13:NM	1(1)	Negative	1,2
1	−	1,2	1	Mult:U	Negative (1)	Negative	1
1	−	1,2	1,2	—:M	1(1)	Negative	1,2

[a] Shown within parentheses is the PCR result using primers of Gannon et al. (12).

primers of Gannon et al. (12). None of these 11 strains was primed with the primer set of Karch and Meyer (22). Southern hybridization of the isolate was positive for 8 of 11 strains, and all were positive for VT-1. Nevertheless, these 11 samples were all positive on direct PCR of stool samples, colony blot hybridization, and FVT testing.

Serotyping and serogrouping showed that a number of isolates are in previously recognized groups that produced VT, but that several strains were nontypeable.

Of the remaining 20 samples which were FVT positive only, 7 were positive for VT-1, 5 were positive for VT-2, and 8 were positive for both VTs; 1 sample positive for FVT-1 and FVT-2 was positive for VT-1 by PCR. The titers of VT-1 and VT-2 on FVT testing were similar when samples from patients with *E. coli* O157:H7 infections, non-O157:H7 *E. coli* infections, or only FVT positivity were compared. Median titers ranged from 8 to 16 in all groups (range, 4 to >256).

By using the guidelines of Galen and Gambino (11) and assuming that colony blots and/or organism recovery are "gold standards" (thereby assuming that PCR-positive samples from culture-negative and colony blot-negative stool samples are false positives), the sensitivity, specificity, positive predictive value, and negative predictive value of PCR for VTEC are 94.6, 97.0, 91.5, and 98.2%, respectively. Results were similar for the detection of stool samples containing VT-1- and/or VT-2-producing organisms. By using FVT as a gold standard for identifying disease, the sensitivity, specificity, positive predictive value, and negative predictive value for the PCR assay are 71.1, 97.2, 93.1, and 86.5%, respectively.

DISCUSSION

The objective of the study described here was the direct detection of VT gene sequences in stool specimens. Direct PCR of untreated stool samples has been used to detect *Shigella* and enteroinvasive *E. coli* (10), enterotoxigenic *E. coli* (18), rotavirus (14, 15), and Norwalk virus (20). However, application of PCR for the direct detection of pathogens in stool specimens has been problematic because of a lack of sensitivity and/or specificity. It has been suggested that DNA extracted from stool contains inhibitors such as bilirubin and bile salts which make amplification difficult (38). Different strategies have been used to overcome these difficulties, including dilution of stool samples before amplification (1, 39), booster PCR (33), immunomagnetic separation of organisms from stool samples (38), or chromatographic purification of viral RNA (39).

Previous studies have shown that PCR is useful in confirming the presence of VT genes in VTEC strains recovered from clinical specimens from patients with hemorrhagic colitis or HUS. One of the earliest PCR methods for the detection of VT genes in clinical isolates of *E. coli* used a single pair of primers which was partly homologous to both VT-1 and VT-2 genes with a one- to two-nucleotide difference between either VT gene and the primers (22). Other investigators have used multiple primer pairs to identify VT-1 and the different VT-2 variants (4, 19, 21, 30, 31, 37).

Direct detection of VTEC from stool specimens has been demonstrated in two other studies. Brian et al. (5) were able to detect a single bacterial genome in stool spiked with indicator VTEC organisms. DNA was extracted directly from stool by methods similar to those used in the present study but had to be diluted 100-fold before PCR was successful (5). However, PCR analysis of *E. coli* O157:H7-positive stools by this method gave positive results in only 4 of 18 instances. In another study (29), PCR detection of VTEC in stool involved inoculating feces into broth and amplifying DNA prepared from 4-h cultures. This method was able to detect 10 CFU/ml against a background of $>10^9$ organisms. Nearly 50% of all samples from otherwise healthy infants or those with diarrhea were positive for VT genes by PCR. Colony hybridization for the recovery of VTEC from some of the PCR-positive samples failed to detect any positive isolates. It is unclear whether false-positive samples accounted for these results.

Our initial approach to direct PCR of stool samples was to inoculate 0.1 g of spiked stool [either *E. coli* C600 (H19B) or *E. coli* C600 (933W)] to BHI broth and allow 2 to 4 h of growth. The boiled suspension was then subjected to PCR. This method was inconsistently sensitive, with thresholds of detection varying from 10^3 to 10^7 CFU per 0.1 g of stool among tests. Moreover, over half of the tests were negative when different stool samples were spiked. Subsequent testing by the spin-wash method in the present study showed consistently positive results.

In order to overcome the problem of inhibition of DNA priming, we used low-speed centrifugation to first remove particulate matter from the stool suspension. The resulting supernatant containing the endogenous stool flora was then subjected to high-speed centrifugation to recover the bacterial pellet. Washing of the bacterial pellet diluted inhibitory substances and allowed for the extraction of DNA from this pellet.

The assay was able to detect 10^2 to 10^4 CFU per 0.1 g of stool for VT-1-containing organisms but only 10^7 CFU per 0.1 g of stool for VT-2-containing organisms. We are uncertain why there is a difference in sensitivity between VT-1 and VT-2 detection by this method. Thresholds of detection were similar between the *E. coli* C600 lysogens and a clinical isolate. Admittedly, additional wild strains could have been tested in the sensitivity experiments. Concordance between tests is a measure of sensitivity. The 78% concordance of colony blot and PCR positivity suggests that the apparent high detection threshold for VT-2 is not clinically relevant in most cases to the point where the assay is insufficiently sensitive. Comparing PCR of stool samples with colony blots for 36 samples, 3 were positive on colony blots but negative on PCR for VT-2. The contribution of viable and nonviable organisms and microcolonies may mean that DNA amounts are higher in infected stools than in spiked stools.

Nevertheless, a number of factors could account for the difference between the 94% correlation of PCR to the identification of stool samples containing VT-producing *E. coli* by other means and the 78% direct concordance by toxin type between PCR of stool samples and colony blot results. Factors that could account for the difference include selection of only one colony blot-positive colony to represent all isolates in the stool in situations in which coinfection could exist, variable limits of detection of VT-1 and VT-2 genes by PCR, or technical difficulties in amplifying the gene in stools. One additional factor accounting for discrepancies in concordance was the testing by PCR several months after the colony blot and FVT tests.

Concordance between gene positivity by PCR or colony blots and FVT or in vitro production of toxin was in the 50% range. Differences in gene positivity and expression of toxin exist. The pathogenic potential of strains of *E. coli* that were recovered from FVT-positive stool samples, that were selected by the colony blots of Newland and Neill (27) and by direct PCR of stool samples, that were primed with the primers of Gannon et al. (12) (11 of 11) or Pollard et al. (30) (8 of 11), and that reacted by Southern blot analysis (8 of 11) but that were not primed by the primer set of Karch and Meyer (22) is unclear. The majority of these organisms were recovered from patients with prolonged undifferentiated diarrhea. At present it is not clear what conditions are required for toxin expression in vivo and in vitro.

Stools containing FVT which were culture and colony blot negative for VTEC were also negative by PCR. This indicates that FVT may persist in the absence of a recoverable pathogen. Karmali et al. (24) demonstrated that FVT is often present in a patient's stool, despite the inability to recover VTEC in cultures. In contrast, organisms were rarely isolated in the absence of FVT. It is unclear why FVT persists after the clearance of the organism from feces. It is postulated that FVT may be released into the lumen by enteroadhering organisms on the mucosal surface or that tissue reservoirs may account for the persistence of the toxin. Alternatively, PCR may not be sufficiently sensitive to detect VTEC in this situation because of low numbers of organisms. Finally, it is possible that VT-positive organisms could lose PCR positivity because of instability of VT gene sequences on serial passage of isolates (22a) and could account for the persistence of FVT with negative colony blot and PCR results. However, samples were frozen and not serially passaged in the present study. Loss of bacteriophage could also account for genetic instability. Testing for FVT remains an important determinant of VTEC disease especially in epidemiologic studies.

It has been shown that in cases of HUS, fewer than 10% of *E. coli* isolates in fecal samples may be VT positive (35). In the present study, the majority of colonies (75 to 90%) selected for colony blot probing were positive if the stool contained VTEC organisms. Because of the high proportion of positive colonies in positive samples, it is unlikely that the colony blot positivity rate would have been different had we picked 5 to 15 representative colonies instead of the 50 used in the present study. It is possible that the results of pathogen testing are different in acute diarrheal disease investigations in comparison with the results when cases of HUS are investigated.

Although PCR is not more sensitive than colony blots for the detection of organisms, colony blots are labor-intensive and do not provide a rapid diagnosis. The PCR assay described here can be completed within 8 h and can provide a rapid screen that can be used to diagnose infection caused by VTEC serotypes. PCR will also detect all serotypes and, compared with culture methods alone, will detect VTEC in stools missed by culture on SMAC. PCR for VTEC in stool samples may provide a rapid method of screening for VTEC in epidemiologic and clinical efficacy studies.

In summary, direct PCR of stool samples from patients with diarrhea detected 34 of 36 (94%) of the samples positive by colony blots and FVT testing. PCR detected VTEC in 3% of 145 samples that were presumed to be negative on the basis of negative *E. coli* O157:H7 cultures or FVT testing. Although promising, the validity of the method with these and other primers will need to be tested in prospective studies. At present it appears that detection of VT in fecal filtrates in combination with PCR or some other gene-based detection method will be required for the definitive diagnosis of infection.

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