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CONCISE COMMUNICATIONS

Improved Detection of Enterotoxigenic *Escherichia coli* among Patients with Travelers' Diarrhea, by Use of the Polymerase Chain Reaction Technique

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This study sought to determine whether a specific polymerase chain reaction (PCR) for enterotoxigenic *Escherichia coli* (ETEC) toxins after chaotropic extraction of DNA from stool would increase the detection of ETEC over that of conventional oligonucleotide probe hybridization of 5 *E. coli* colonies per stool sample (a standard method). By DNA hybridization, 29 (21%) of 140 patients were positive for ETEC, and 59 (42%) of 140 were positive for ETEC when PCR was used. Sensitivity of the PCR assay was confirmed through spiked stool experiments to be ~100–1000 ETEC colonies per sample. Specificity of the assay was determined by showing an absence of ETEC by the PCR technique in a subgroup of 48 subjects and by confirming the presence of ETEC DNA of positive samples by dot blot procedure. PCR technique detected significantly more ETEC infections in these subjects than did the hybridization method ($P < .0001$).

In 1971, enterotoxigenic *Escherichia coli* (ETEC) were shown to cause diarrhea in healthy volunteers [1]. ETEC cause diarrhea through the action of heat-labile (LT) and heat-stable (ST) enterotoxins. ETEC strains may express only LT or ST or may express both LT and ST [2]. Diagnosis of ETEC infection relies on biologic or immunologic detection of the ST or LT in fecal *E. coli* isolates or on identification of the genes encoding for the toxins. It is common for laboratories studying ETEC to test for the presence of toxin genes by oligonucleotide probe hybridization of 5 *E. coli*–like colonies from the stool sample of each patient [3, 4].

Detection of ETEC by a multiplex polymerase chain reaction (PCR) assay in stool specimens directly processed with a chaotropic solution and a DNA glass matrix has been reported to have greater sensitivity than other methods used to extract nucleic acid from stools [5]. The binding of DNA to glass particles in the presence of chaotropic agents is well documented [6]. The chaotropic agent guanidine thiocyanate (GuSCN) is a powerful agent for purifying and detecting both DNA and RNA, apparently because of its ability to lyse cells combined with its ability to inactivate nucleases.

This study compared ETEC toxin detection from specimens of patients with travelers' diarrhea by 2 methods: oligonucleotide probes for LT and ST hybridized with 5 *E. coli*–like colonies per stool sample (our standard assay) and a multiplex PCR of DNA extracted from stool specimens by use of the chaotropic DNA glass matrix method, which simultaneously detects the genes encoding for LT and ST.

Methods

Clinical Specimens

We studied 140 stool samples from patients with diarrhea who had traveled to Guadalajara, Mexico (70 subjects), and Montego Bay, Jamaica (70 subjects), during the summer of 1997. Stool samples from 48 healthy Americans living in Houston were used as negative controls. Patient stool specimens were subjected to microbiologic analysis in our field laboratories in Guadalajara and Montego Bay. Five individual *E. coli*–like colonies and an aliquot of stools from each subject were immediately stored at −20°C until processed.

DNA Hybridization Assay

As previously described, 5 individual *E. coli*–like colonies from each stool sample were grown in the field laboratory and fixed to Whatman 541 filters (Whatman, Clifton, NJ). Then they were hybridized with ST and LT oligonucleotides that were labeled by using T4 polynucleotide kinase and [^{32}P] ATP [7].

Preparation of Stool Specimens for PCR and DNA Purification

Stool lysis. DNA was extracted from feces by a modification of a procedure that uses a chaotropic glass matrix method to obtain

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DNA for ETEC detection by PCR [5]. In brief, 100 mg of a thawed stool sample was suspended in 1.5 mL of normal saline (0.85%) in microcentrifuge tubes. To remove debris from the stool, tubes were centrifuged at 183 *g* for 1 min. The supernatants were transferred to new tubes and centrifuged at 16,000 *g* for 5 min. The supernatants were then discarded; the pellets were washed in 1 mL of PBS and centrifuged as above. The supernatants were discarded once again, and the pellets were resuspended in 0.6 mL of chaotropic solution (2.65 *M* guanidine thiocyanate, 5 m*M* dithiothreitol, 0.5% Tween 20, 0.15 *M* sodium acetate, 25 m*M* sodium citrate, 0.36% ammonium bromide, and 0.28 *M* sodium chloride, final pH 7.0) and were incubated at 65°C for 20 min.

DNA purification. After incubation with chaotropic solution, 50 μL of resuspended glass matrix (GlasPac/GS; National Scientific Supply, San Rafael, CA) was added and incubated for 15 min at room temperature with continuous mixing. The suspension was centrifuged at 16,000 *g* for 1 min, and the supernatant was discarded. The matrix was resuspended in 1 mL of wash buffer (GlasPac/GS) and centrifuged at 16,000 *g* for 1 min. This wash step was repeated twice. The matrix pellet was dried at room temperature for 5 min, and the bound DNA was eluted by incubation with 100 μL of elution solution (10 m*M* Tris-HCl [pH 8.0], 0.1 m*M* EDTA) at 50°C for 10 min with periodic mixing. The suspension was centrifuged at 16,000 *g* for 2 min, and the eluted DNA (supernatant) was carefully transferred to a new tube for PCR amplification.

PCR amplification. Oligonucleotide primers for LT and ST were selected on the basis of previously published sequences [5]. The final amplification mix contained 90 μL of PCR mix (10 m*M* Tris-HCl [pH 8.3]; 50 m*M* KCl; 2 m*M* $MgCl_2$; 100 μg/mL gelatin; 5% glycerol; 1 μM [each] dATP, dCTP, dGTP and dTTP; and 2.5 U of AmpliTaq polymerase [Perkin-Elmer, Norwalk, CT]), 25 pmol of each of the 4 primers, and 10 μL of stool DNA solution. The reaction mixtures were heated to 50°C for 2 min and 95°C for 5 min and were subjected to 40 cycles (95°C for 45 s and 50°C for 45 s) and finally to an extension at 72°C for 10 min in a DNA thermal cycler (Perkin-Elmer).

Detection of amplified products. We analyzed 20 μL of the amplified PCR products by 3% agarose electrophoresis gels in TBE buffer (89 m*M* Tris-HCL [pH 8.3], 89 m*M* boric acid, and 2.5 m*M* EDTA). In addition to the 1-kb DNA molecular weight marker (Gibco BRL, Gaithersburg, MD), 2 DNA controls were run simultaneously with the samples. A positive control, an amplified PCR product from a stool sample spiked with H10407 ETEC strain, and a negative control, a PCR product from the stool of a healthy noninfected person spiked with *E. coli* strain JCP88, were included in the assay.

Dot blot analysis was done for a subset of 50 samples (25 from Mexico, 25 from Jamaica). Five microliters of PCR products was dot blotted onto nitrocellulose membranes, denatured with NaOH/NaCl, and cross-linked with UV light. The membranes were hybridized with [^{32}P] ATP-labeled oligonucleotide probes for ST and LT, which were selected from published sequences, and visualized by autoradiography [5].

Sensitivity test. To assess the sensitivity of the PCR method, we performed experiments in which 100-mg samples of stools were spiked with varying concentrations (10^1–10^7 cfu/g) of ETEC strain H10407, following MacFarland's opacity standard.

Statistical Analysis

McNemar's exact χ^2 test was used for correlated proportions of the 2 methods. The α level was set at 0.05.

Results

DNA hybridization. Of the 140 stool samples analyzed, 29 (21%) were positive for toxin genes by this method (table 1).

PCR products. The 140 PCR products from the same patients were analyzed as described above using a 3% agarose gel and visualized with ethidium bromide. Positive samples yielded bands at ~450 bp for LT and at ~190 bp for ST, as reported elsewhere [5]. By the multiplex PCR, 59 (42%) of 140 stool samples were positive. Of the 29 hybridization-positive isolates, 28 were positive by PCR (table 2). Fifty randomly selected PCR products were analyzed by dot blot oligonucleotide probes specific for ST and LT. Samples positive by PCR were also positive using these specific DNA probes. The PCR method detected significantly ($P < .0001$) more ETEC in stool samples than the DNA hybridization method. When the 2 methods were compared regarding detection of toxin-encoding genes for LT and ST, the PCR method detected more LT and ST genes than the hybridization method (table 1).

Sensitivity test. PCR methodology detected ETEC toxin DNA (ST and LT) when ETEC concentrations in the stools were 100–1000 organisms/g stool.

Control samples. Stool samples from 48 healthy American volunteers without diarrhea were negative for the presence of ETEC when analyzed by the hybridization and PCR methodology.

Discussion

These results show that this PCR method is significantly more sensitive than a standard DNA hybridization technique in detecting ETEC in stools. Several factors make the multiplex PCR chaotropic DNA glass matrix assay a more sensitive, rapid, and less laborious technique for ETEC detection than the hybridization assay. The PCR method eliminates the need for a culture step, which depends on the presence of viable organisms in the stool. Microorganisms may be difficult to grow when their number is low and probably impossible if samples have been repeatedly frozen/thawed or kept frozen for months or

Table 1. Distribution of ETEC toxin–encoding genes as detected by PCR versus DNA hybridization in 140 stool samples from patients with travelers' diarrhea in Mexico and Jamaica.

Method of detection	No. (%) of stools with positive results		
	Heat stable (ST)	Heat labile (LT)	ST/LT
DNA	14 (10)	8 (6)	7 (5)
PCR	25 (18)	16 (11)	18 (13)

NOTE. ETEC, enterotoxigenic *Escherichia coli;* PCR, polymerase chain reaction.

Table 2. Comparative results of polymerase chain reaction (PCR) versus DNA hybridization in 140 stool samples from patients with travelers' diarrhea in Mexico and Jamaica.

Test result	PCR positive	PCR negative	Total
DNA positive	28	1	29
DNA negative	31	80	111
Total	59	81	140

years. In the present study, stool samples that were frozen at −20°C for 6 months were analyzed by PCR after thawing with good results, and 28 (97%) of 29 fresh stool samples positive by DNA hybridization were detected by PCR. DNA extraction from stools using the chaotropic glass matrix method permits the isolation of most of the DNA present in a sample, thereby diminishing the problem of intrinsic PCR inhibitors.

Detection of shigellae, ETEC, and *Campylobacter* species by multiplex PCR using this DNA extraction procedure has been reported [8]. The simultaneous identification of several pathogens from the same sample by use of this technology could be of great advantage in studies of acute diarrhea. Furthermore, the chaotropic DNA glass matrix method has a greater sensitivity than other techniques, such as conventional total nucleic acid extraction and ethanol precipitation [5]. The method used in the present study detected ETEC toxin DNA when ETEC concentrations were 100–1000 organisms/g stool. This is similar to the level of ETEC organisms (800) identified by the PCR-chaotropic method in another study [8].

The exquisite sensitivity of PCR can result in false-positive reactions due to contamination or carryover. Therefore, in this study, we physically separated the main PCR steps: DNA stool extraction, preparation of reaction mixture, and manipulation of PCR products [9]. Thus, the detection of increased ETEC toxin is most likely due to increased sensitivity and not to false-positive results. This is supported by the fact that the 40 ETEC-negative controls remained negative when analyzed by the same method. The fact that 28 of 29 fecal samples identified as ETEC-positive by DNA hybridization were positive by PCR assay also suggests that the PCR assay is sensitive in detecting ETEC from fecal samples. When randomly selected PCR products of 50 samples were analyzed by hybridization with oligonucleotide probes, the samples identified as positive by PCR were also positive by the former method, offering further evidence that the PCR was specific.

One problem related to the diagnosis of an infection based only on the detection of DNA is the high sensitivity of the PCR assay. It is also possible that viable or nonviable ETEC were present and detectable in stool from environmental (food, water) contamination of the gastrointestinal tract of the patients in the absence of symptomatic ETEC infection.

The finding of ETEC infection in persons who, without PCR study, would have an undetected enteropathogen may explain, in part, our results showing a benefit of antimicrobials in shortening illness in cases of nonspecific illness [10]. In this study, which employed a PCR procedure, we found a higher percentage of ETEC infection in a group of patients with travelers' diarrhea than we found by a less sensitive DNA hybridization method. Thus, PCR may help to establish the true importance of ETEC in various populations (travelers, local populations in endemic areas, and foodborne outbreaks).

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