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Multiplex PCR Assay and Simple Preparation Method for Stool Specimens Detect Enterotoxigenic *Escherichia coli* DNA during Course of Infection

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Infection with enterotoxigenic *Escherichia coli* (ETEC) is a common cause of diarrhea among travelers and residents of developing countries. ETEC produces either a heat-stable toxin or a heat-labile toxin, or both, encoded by plasmid-borne ST and LT genes, respectively. Diagnosis of infection with this subclass of *E. coli* can be performed with oligonucleotide hybridization probes; however, the sensitivity and specificity of this method are insufficient. A nonradioactive multiplex PCR assay that provides a sensitive and specific method for detecting the presence of either or both toxin genes has been developed. A simple procedure that removed inhibitors of the PCR while efficiently releasing ETEC DNA from stool specimens for subsequent amplification was used. The results for samples from a human volunteer study of ETEC infection indicated that this method of sample preparation results in greater clinical sensitivity than conventional total nucleic acid extraction and ethanol precipitation. Detection of ETEC by a multiplex PCR assay in stool specimens directly processed with a glass matrix and chaotropic solution had greater sensitivity than culture.

Escherichia coli strains that produce enterotoxins cause significant numbers of cases of childhood diarrhea in developing countries and gastroenteritis among travelers. The pathogenicity of enterotoxigenic *E. coli* (ETEC) is due to the production of heat-labile enterotoxin (LT), heat-stable enterotoxin (ST), or both toxins. These genes are plasmid encoded and can be present on the same multicopy plasmid. LT consists of two subunits, and the LT gene sequences from a variety of human and animal isolates are highly homologous (24). ST is classified into two main groups: STI and STII. The sequences of several alleles of the *estA* genes encoding STI have been described: *estA1* originally isolated from *E. coli* of animal origin encodes ST_p, and *estA2-4* from *E. coli* of human origin encodes ST_h. Sequence homology between the *estA1* allele and the other alleles is only about 70%, with the highest degree of conservation found in the region that encodes for the mature STI peptide. Strains producing either or both types of STI have been found to be human pathogens.

No biochemical or serological markers exist to differentiate enterotoxigenic strains of *E. coli* from nontoxigenic strains. Detection of these strains has depended on demonstration of toxin production by specific bioassays and enzyme-linked immunosorbent assays or on the presence of the DNA sequences encoding the toxins. DNA probes were widely used to study ETEC; however, cross-reactivity and a requirement for enrichment of viable cells from the background level of dead cells made the development of PCR-based tests for the detection of ETEC desirable. Several assays that detect ETEC have been described (2, 5, 7–9, 15, 17, 22, 23). In the study described here, we used two sets of primers to simultaneously detect the genes encoding LT, ST_h, and ST_p in order to detect all types of ETEC in a single multiplex reaction.

Detection of enteric bacteria in fecal specimens from infected humans and animals by PCR has been relatively laborious and insensitive because of the large amounts of inhibitors to PCR such as bile salts, bilirubin, urobilinogens, polysaccharides, and large amounts of irrelevant DNA. The direct addition of fecal specimens to PCR assays was only possible when the specimens were made dilute, resulting in a low detection limit (1, 9). PCR analysis of the *E. coli*-like bacteria in stool specimens inoculated onto agar plates had good sensitivity, but it required overnight incubation (19). Techniques that concentrate the pathogen or total nucleic acids and that remove inhibitors of PCR have shown promise when they are applied to fecal specimens. Immunomagnetic capture of bacteria (16) requires the availability of specific antiserum or a monoclonal antibody and a bacterial surface antigen common to all target pathogens. Commercially available matrices have been used to purify total nucleic acids obtained by classical multistep procedures involving organic extractions from fecal specimens (4, 10). In the present study, a glass matrix and a chaotropic solution were used to directly purify ETEC DNA from clinical fecal specimens for subsequent PCR detection of ETEC.

MATERIALS AND METHODS

Bacterial strains and plasmids. ETEC strains isolated from patients with diarrheal disease were obtained from the Center for Vaccine Development, University of Maryland School of Medicine (including strains H10407, E23666A, and M408C1), and Stanford University (including strains 2539-C1, 2513-72, and 5203-70). The presence of the appropriate toxin genes was verified by oligonucleotide probe hybridization. Plasmid pWD600 containing a 5.4-kb *Pst*I fragment with the LT gene of ETEC strain H74-114 (6) was provided by Walter Dallas, Burroughs Wellcome Co.

Clinical specimens. Eleven healthy adult volunteers were housed in the isolation ward at the Center for Vaccine Development, University of Maryland School of Medicine, and were provided with a varied hospital diet. Each adult was fed a dose of 1.7×10^9 CFU of ETEC H10407 strain (O78:H11), which produces LT, ST_h, and ST_p, after gastric neutralization with 2 g of sodium bicarbonate in water. Following 4 days of diarrhea, every volunteer with diarrhea was treated with antibiotics to cure the infection. Every stool was collected from the volunteers (including a sample prior to inoculation), numbered sequentially, and cultured for the presence of the inoculation bacterium. ETEC H10407 was quantitated in three stools per day from each volunteer following serial dilution in saline. The ETEC H10407 bacteria were differentiated from other *E. coli* strains by agglutination with both anti-O78 and anti-CFA I serum (no *E. coli* strain agglutinated with only one antiserum). All microbiological methods were

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performed as described previously (21). Subsequently, all stool specimens were coded and stored at −20°C until the samples were prepared for PCR analysis.

Preparation of stool specimens for PCR analysis. (i) Lysis with a chaotropic solution and purification of DNA by binding to a glass matrix. Approximately 100 mg or 100 μl of thawed stool sample was suspended in 1.5 ml of Dulbecco's phosphate-buffered saline in a microcentrifuge tube, and the tubes were centrifuged at 2,800 × *g* for 1 min to remove the debris. The supernatant was transferred to a new tube, and the tube was centrifuged at 16,000 × *g* for 5 min. The supernatant was discarded, the pellet was resuspended in 0.6 ml of GuSCN binding/lysis buffer (5.3 M guanidine thiocyanate, 10 mM dithiothreitol, 1% Tween 20, 0.3 M sodium acetate, 50 mM sodium citrate [pH 7.0]), and the mixture was incubated at 65°C for 10 min. Fifty microliters of resuspended glass matrix (GlasPac; National Scientific Supply Company, San Rafael, Calif.) was added to the solution, and the mixture was incubated at room temperature for 15 min with 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 (50% ethanol, 10 mM Tris-HCl [pH 7.5], 100 mM NaCl). Following centrifugation as described above, the wash step was repeated twice. The matrix pellet was dried at room temperature for 5 min. Bound DNA was eluted by incubation in 100 μl of 10 mM Tris-HCl (pH 8.0)–0.1 mM EDTA at 50°C for 5 min with periodic mixing. Following centrifugation at 16,000 × *g* for 2 min, the eluate was transferred to a new tube.

(ii) DNA purification by conventional nucleic acid extraction. Bacterial DNA was extracted from 100 mg or 100 μl of thawed stool sample by treatment with lysozyme and sodium dodecyl sulfate (SDS), digestion with proteinase K, and then ethanol precipitation as described by Frankel et al. (7). For comparison, a more purified extract of DNA was prepared by treating the crude extract with RNase A and performing multiple extractions with phenol-chloroform prior to ethanol precipitation.

PCR amplification. The oligonucleotides used to amplify a 450-bp segment of the LT-A gene were an upstream primer TW20 (5′-GGCGACAGATTATAC CGTGC-3′; nucleotides 4 to 23 in reference 24; primer LTPr1 described by Frankel et al. [7]) and a downstream primer JW11 (5′-CGGTCTCTATATTC CCTGTT-3′; nucleotides 443 to 424). The oligonucleotides used to amplify a 190-bp segment of the STI genes (*estA1-4* alleles) were an upstream primer JW14 (5′-ATTTTTMTTTCTGTATTRTCTT-3′; M = A or C and R = A or G; nucleotides 22 to 43 in reference 12) and a downstream primer JW7 (5′-CAC CCGGTACARGCAGGATT 3′; nucleotides 212 to 193). Amplification of the LT and ST gene targets was conducted in a single 100-μl reaction volume containing 50 μl of diluted DNA. The final amplification reactions contained DNA in 10 mM Tris-HCl (pH 8.3)–50 mM KCl–2 mM $MgCl_2$–100 μg of gelatin per ml–5% glycerol–200 μM (each) dATP, dCTP, dGTP, and dUTP–2.5 U of *AmpliTaq* polymerase (Perkin-Elmer, Norwalk, Conn.)–1 U of uracil-*N*-glycosylase (Perkin-Elmer)–25 pmol of each of the four primers. The reaction mixtures were heated to 50°C for 2 min and 95°C for 5 min and were then subjected to 40 cycles of 95°C for 45 s and 50°C for 45 s and a final extension at 72°C for an indefinite period of time (at least 10 min) in a DNA thermal cycler (Perkin-Elmer).

Detection and analysis of amplified product. Ten microliters of the amplification reaction mixtures were analyzed by gel electrophoresis in 3% NuSieve agarose–0.5% SeaKem agarose gels in TBE buffer (89 mM Tris-HCl [pH 8.3], 89 mM boric acid, 2.5 mM EDTA). For dot blot analyses, 10 μl of the amplified DNA was denatured with NaOH, transferred to a membrane, and cross-linked with UV light. The sequences of the hybridization probes are as follows: LT probe RDR91, 5′-AATTTTGGTGTGATTGATGA 3′ (nucleotides 391 to 410); ST_h probe RDR250, 5′-GTCCTGAAAGCATGAATAGT-3′ (nucleotides 145 to 165); ST_h-ST_p probe RDR283, 5′-GTCTTCAAAAGARAAAATYACAC T-3′ (Y = C or T; nucleotides 78 to 101), and ST_p probe RDR387, 5′ ATCA-GAAAATATGAACAACACATT-3′ (nucleotides 147 to 170). The membranes were prehybridized in a solution containing 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA [pH 7.4]), 0.5% SDS, and 0.5% dextran sulfate at 42°C for 30 min. The membranes were hybridized by the addition of horseradish peroxidase-labeled probe to a final concentration of 3 nM, and the mixture was incubated for 30 min at 42°C. Following incubation with probe, the membranes were washed with 1× SSC (1× SSC is 0.15 M NaCl and 15 mM citrate [pH 7.0])–0.1% SDS at 45°C for 15 min. Bound probe was detected with the Enhanced Chemiluminescent Detection kit (Amersham, Arlington Heights, Ill.). The analysis was performed with the individual probes or by combining individual probes into pools.

PCR analysis of clinical stool samples. ETEC H10407-containing stool samples were identified as PCR positive by detection of LT, ST_h, and ST_p DNAs in duplicate reactions following gel electrophoresis and dot blot hybridization. Extracts that were negative for ETEC DNA by PCR analysis were analyzed for the presence of PCR inhibitors by the addition of 10^4 copies of LT-containing pWD600 plasmid DNA.

RESULTS

Detection and typing of ETEC clinical isolates by PCR. Oligonucleotide primers were designed to simultaneously amplify the LT-A and STI genes of human and pig ETEC isolates in a single PCR tube. Primers designed by Frankel et al. (7) were extensively modified to improve amplification efficiency and specificity in the multiplex assay and to include ST_p. ST primers JW7 and JW14 were synthesized with degeneracies to efficiently amplify both the ST_h and ST_p gene sequences. The LT primers (TW20 and JW11) and the ST primers (JW14 and JW7) specifically amplified the appropriate genes from the DNAs of 32 different clinical ETEC isolates. A detection limit of DNA from 10 to 100 organisms was obtained following agarose gel electrophoresis and ethidium bromide staining. Oligonucleotide probes designed to discriminate among the amplification products of the multiplex assay independently detected the presence of the LT gene product (RDR91), the ST_h gene product (RDR250), the ST_p gene product (RDR387), and both ST gene products (RDR283). All 32 ETEC isolates were accurately typed by this method (examples are shown in Fig. 1).

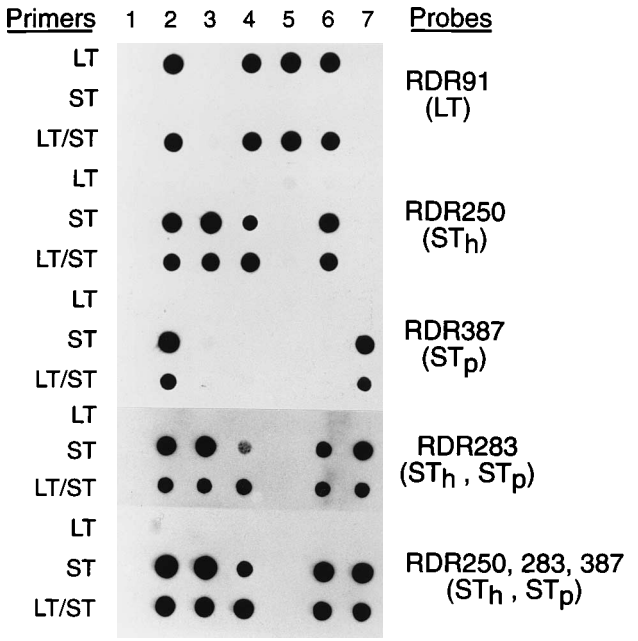

FIG. 1. Dot blot hybridization analysis of ETEC clinical isolates. A total of 10 pg of ETEC DNA was subjected to PCR amplification with either the LT primers (JW11 and TW20), the ST primers (JW7 and JW14), or all four primers (LT/ST) and was detected with the hybridization probes. Lanes: 1, no DNA; 2, H10407 (LT, ST_h, ST_p); 3, 5203-70 (ST_h); 4, 2513-72 (LT, ST_h); 5, 2539-C1 (LT); 6, M408C1 (LT, ST_h); 7, E23666A (ST_p).

Detection of ETEC DNA in clinical stool specimens by PCR. Sequential stool specimens were obtained from 11 human volunteers inoculated with ETEC H10407. The samples were collected prior to infection, during infection, and following antibiotic treatment. Nucleic acids were extracted by a conventional technique (7) from 65 coded stool specimens representing 1 specimen from each volunteer per day. A 1:9,000 dilution of extracted DNA was required to reduce the concentration of inhibitors to the PCR, resulting in PCR analysis of 5.5 μg of stool per reaction. Specimens that were initially negative for ETEC DNA by PCR and dot blot hybridization were reassayed for ETEC DNA by using other dilutions and were also tested for inhibitors of PCR by the addition of target DNA. In general, stool specimens that were formed or semisolid required further dilution compared with liquid specimens obtained from patients with diarrhea. With this protocol, 26 of 65 of the specimens did not contain ETEC DNA, as detected by PCR

analysis and dot blot hybridization (Table 1). Twelve ETEC-containing specimens detected by culture were not detected by this initial PCR analysis by using dilutions ranging from 1:50 to 1:15,000 (Table 1).

Comparison of methods for extraction of DNA from clinical stool specimens. Prior to breaking the code, two additional methods for extracting DNA from stool specimens were examined for their efficiency of recovery of ETEC DNA and their ability to inhibit PCR amplification by using a subset of the clinical specimens described above (including several specimens that contained a high concentration of PCR inhibitors). A comparison was made between (i) the conventional treatment with lysozyme, detergent, and proteinase K and then ethanol precipitation used in the previous section (method NC), (ii) conventional nucleic acid extraction and then digestion with RNase A, extraction with phenol-chloroform, and ethanol precipitation (method P), and (iii) lysis with guanidine thiocyanate and then adsorption and elution of the DNA from a glass matrix (method G). When 10-fold serial dilutions of the extracts obtained from three clinical ETEC-containing stool specimens were analyzed by PCR and gel electrophoresis (Fig. 2), a 1:10 or 1:50 dilution was required before the extracts prepared with the glass matrix produced the appropriate LT and ST gene products. No ETEC DNA was detected from the phenol-chloroform-extracted material until a 1:1,000 dilution was performed. Target ETEC DNA appeared to be limiting in the 1:1,000 dilution of extracts prepared by methods P and G, as demonstrated by the variabilities seen in duplicate amplification reactions. The extracts prepared by the conventional nucleic acid technique required further dilution (at least a 1:9,000 dilution) to reduce the level of PCR inhibitors until ETEC DNA was detected. The presence of inhibitors to PCR in the dilutions was distinguished from the absence of target DNA by the addition of 1 ng of genomic ETEC H10407 DNA prior to PCR analysis.

When ETEC H10407 bacterial cells grown in culture were spiked into control stool specimens, a detection limit of 10^5 bacteria per g of stool was obtained by the glass matrix method of sample preparation (data not shown). Further improvement in the detection limit was obtained by reducing the centrifugal force of the initial centrifugation (to remove debris from the stool specimen) from 2,800 to 183 $\times$ *g* and adding hexadecyltrimethyl ammonium bromide and sodium chloride, at final concentrations of 0.73% and 0.57 M, respectively, to the lysed specimens prior to the addition of the glass matrix.

Analysis of clinical stool specimens following extraction with a glass matrix. The glass matrix protocol was used to prepare DNA extracts from the 26 specimens that were negative for ETEC DNA by PCR and dot blot hybridization in the initial analysis described above. Dilutions of 1:50 were analyzed by PCR and dot blot hybridization, and the remaining PCR-negative specimens were shown to be amplifiable at that dilution by the addition of exogenous DNA. Dilutions of 1:10 were also tested if noninhibitory to PCR. ETEC DNA was detected in 13 specimens only when the glass matrix protocol was used to extract DNA; 12 of these specimens were positive for ETEC by culture. The final results for PCR and dot blot hybridization analysis are tabulated in Table 1 along with culture results. Equivalent results were obtained by gel electrophoresis and dot blot hybridization analysis. All culture-positive specimens were detected by PCR analysis, resulting in 100% sensitivity. Four specimens that did not contain ETEC as determined by culture were found to contain ETEC DNA by PCR analysis, and all of these specimens were obtained following the initiation of antibiotic therapy. Thirteen other specimens obtained during antibiotic therapy contained ETEC, as

TABLE 1. Comparison between PCR and culture for the detection of ETEC in clinical stool specimens

Patient no.	Sample no.	PCR result	Culture result	Quantitative counts[a]
1	1-0[b]	−	−	NA[c]
	1-4[b]	+	+	1.0×10^9
	1-7	+	+	1.8×10^8
	1-9	+	+	9.0×10^7
	1-11[b,d]	+	+	NA
	1-12[d]	+	+	NA
2	2-0[b]	−	−	NA
	2-3	+	+	1.6×10^8
	2-15	+	+	2.0×10^8
	2-17	+	+	1.2×10^8
	2-18[d]	+	+	NA
	2-19[d]	+	−	NA
3	3-0[b]	−	−	NA
	3-10	+	+	1.9×10^8
	3-16	+	+	1.5×10^8
	3-17[d]	+	+	NA
	3-18[d]	+	+	NA
4	4-0[b]	−	−	NA
	4-2	+	+	2.0×10^8
	4-5	+	+	2.0×10^8
	4-8	+	+	5.0×10^8
	4-11[d]	+	+	NA
	4-12[d]	+	+	NA
5	5-0[b]	−	−	NA
	5-7	+	+	3.0×10^8
	5-14	+	+	1.7×10^8
	5-16	+	+	1.0×10^9
	5-17[d]	+	+	NA
	5-18[b,d]	+	+	NA
6	6-0[b]	−	−	NA
	6-3[b]	+	+	2.5×10^8
	6-4[b]	+	+	2.8×10^7
	6-5[b]	+	+	1.2×10^7
	6-6[b,d]	+	+	NA
	6-7[b,d]	−	−	NA
12	12-0[b]	−	−	NA
	12-6	+	+	1.0×10^8
	12-9	+	+	1.0×10^8
	12-12[b]	+	+	6.0×10^7
	12-14	+	+	1.0×10^8
	12-17[d]	−	−	NA
13	13-0[b]	−	−	NA
	13-3	+	+	1.4×10^8
	13-6	+	+	1.8×10^8
	13-7	+	+	1.3×10^8
	13-8[d]	+	+	NA
	13-9[d]	+	−	NA
16	16-0[b]	−	−	NA
	16-7	+	+	1.5×10^8
	16-16	+	+	3.0×10^8
	16-19	+	+	2.2×10^8
	16-24[d]	+	−	NA
	16-27[d]	−	−	NA
19	19-0[b]	−	−	NA
	19-2[b]	+	+	7.0×10^5
	19-3[b]	+	+	6.0×10^6
	19-6	+	+	7.5×10^7
	19-8[d]	+	+	NA
	19-10[b,d]	−	−	NA
22	22-0[b]	−	−	NA
	22-2	+	+	1.0×10^8
	22-3[b]	+	+	7.2×10^7
	22-6	+	+	7.0×10^7
	22-8[b,d]	+	+	NA
	22-10[b,d]	+	−	NA
DNA control	H10407, 10 pg	+	NA	NA

[a] Number of *E. coli* H10407 colonies per gram of stool. *E. coli* H10407 was identified by agglutination with anti-O78 and anti-CFA serum.
[b] Negative or inhibitory when prepared by conventional nucleic acid extraction.
[c] NA, not available.
[d] Specimens were collected during antibiotic treatment.

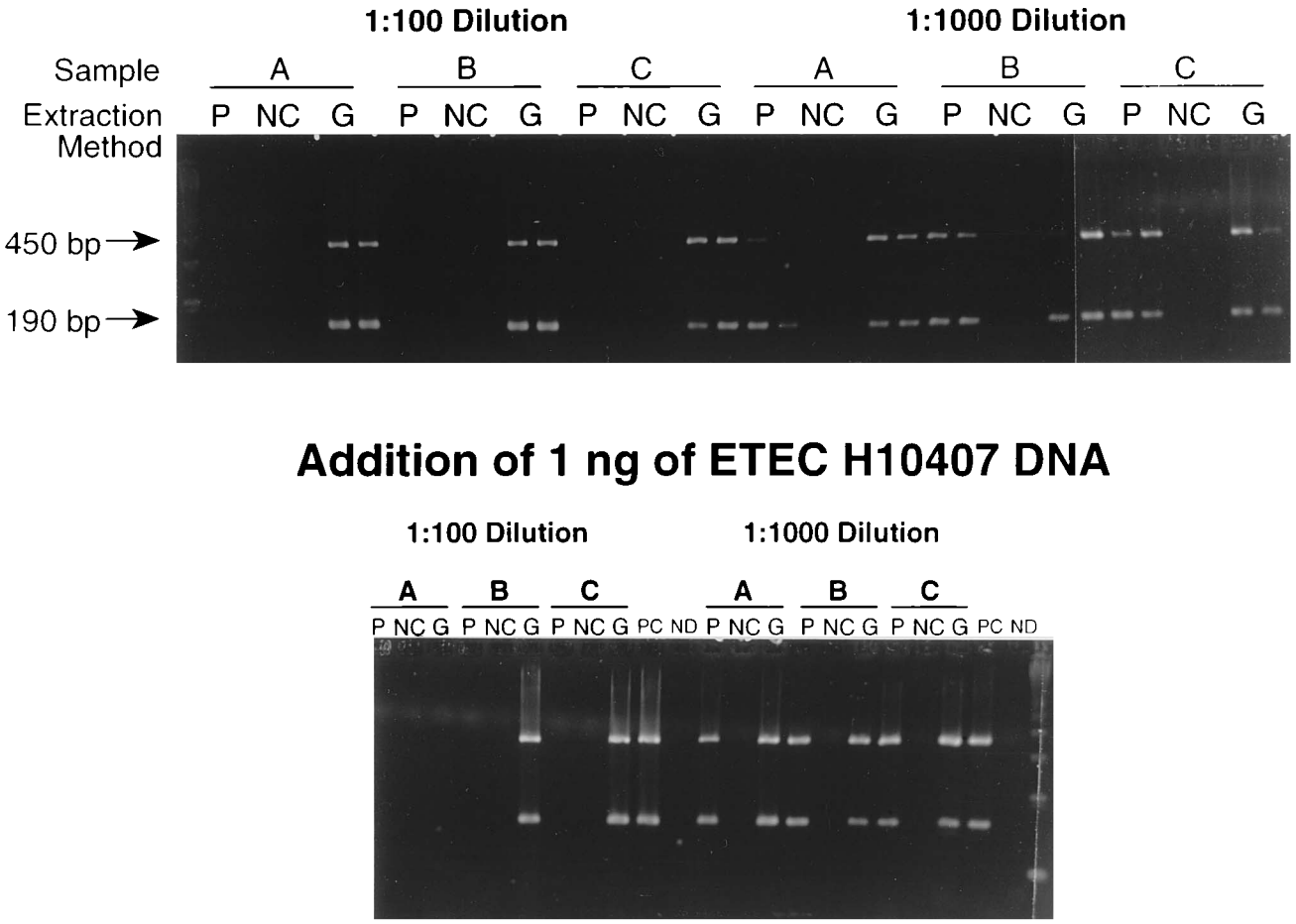

FIG. 2. Recovery of ETEC DNA and PCR inhibitors by three stool sample preparation methods. Total nucleic acids were extracted from three ETEC-containing stool samples, patient sample 22-2 (A), patient sample 22-8 (B), and patient sample 22-10 (C), by different sample preparation methods: P, conventional nucleic acid extraction including extraction with phenol-chloroform; NC, conventional nucleic acid extraction; and G, lysis and extraction of nucleic acid with guanidine thiocyanate and a glass matrix. Dilutions of the extracts were amplified in duplicate by the multiplex ETEC PCR assay to determine DNA recovery. The presence of DNA inhibitors was analyzed by the addition of target ETEC DNA. PC, positive control of 1 ng ETEC H10407 DNA; ND, no DNA added.

detected by both culture and PCR analysis. No ETEC DNA was detected by PCR analysis in the 11 specimens obtained from patients prior to their inoculation with ETEC.

DISCUSSION

The development of a simple, rapid, and efficient protocol for preparing clinical stool specimens for PCR analysis was key to the sensitive and specific detection of ETEC DNA during the course of human infection. When a conventional method for preparing total nucleic acids from stool samples by detergent and protease digestion and then ethanol precipitation was used, the sensitivity of PCR detection compared with the sensitivity of culture was only 74%, whereas the sensitivity was 100% by a glass matrix protocol. The problem with using total nucleic acids prepared by the conventional method was not the recovery of ETEC DNA but the presence of inhibitors of PCR. The level of ETEC DNA in the extracted material had to be high such that the extensive dilution required to reduce the level of inhibitors to PCR still resulted in a detectable level of ETEC DNA. The ability to purify ETEC DNA from inhibitors by the glass matrix protocol resulted in the detection of several culture-positive specimens, whereas dilution of crude extracts failed. This PCR assay had a detection limit of 10 to 100 ETEC organisms; thus, a 1:9,000 dilution of the crude extract required at least 4×10^6 to 4×10^7 cells per g of stool if there was no PCR inhibition. The glass matrix protocol resulted in a detection limit of approximately 10^5 cells per g, as determined by adding cultured cells to stool specimens prior to processing. This detection limit is similar to that reported for PCR assays for ETEC used in concert with immunomagnetic separation of ETEC (9), purification of crude nucleic acid extracts on an ion-exchange resin (14), and the use of crude total nucleic acid extracts (7) and is suitable for detecting clinical infections. However, the sensitivity of the assay for detecting specific DNA sequences in fecal specimens varied between individual specimens in the present study and as noted previously (9, 18). In the present study we found that the levels of inhibitors to PCR, as measured by dilution and the addition of exogenous target DNA, varied significantly between clinical stool specimens. Generally, when the patient suffered from diarrhea, the level of PCR inhibitors was low. The shortened transit time results in reduced levels of dietary material and anaerobes. However, there was not an absolute correlation between the level of ETEC, as determined by quantitative culture, and the level of inhibitors. All specimens obtained prior to ETEC inoculation and many of the specimens obtained following antibiotic therapy to eliminate the infection contained high levels

of inhibitors, but in addition, all specimens from individual 6 also contained high levels of inhibitors.

Stool specimens were scored as positive by PCR for ETEC H10407 when all three target gene sequences (LT, ST_h, ST_p) were present, although no partial results were observed. When PCR was selected as the reference standard for ETEC infection, culture was 92% sensitive. The four PCR-positive, culture-negative stool specimens were obtained during antibiotic therapy to eradicate the ETEC infection. Thus, it is possible that either nonculturable ETEC or free ETEC DNA was detected in these specimens by PCR. Alternatively, PCR had a greater sensitivity for ETEC detection than culture performed without selection for ETEC, and only low levels of ETEC remained in these specimens. In 13 other specimens obtained during antibiotic therapy, ETEC was detected by both culture and PCR. Infection was cleared by 2 days of antibiotic treatment in 7 of 11 volunteers as measured by culture and in 4 of 11 volunteers as measured by PCR.

The selection of oligonucleotide primers located in regions of homology that contained mixed bases at sites of variation between alleles of the *estA* gene resulted in the detection of a wide range of ST-containing clinical ETEC isolates. An alternative to using ST primers with degeneracies is to incorporate deoxyinosine into locations where several bases are expected, as used by Candrian et al. (5). Their upstream primer EC01 is located in the same region as JW14 and performs equivalently (data not shown). When ST primers located in the same general regions as JW7 and JW14 were chosen to be homologous to only the ST_h gene sequence, amplification of DNAs from isolates producing ST_p was absent (2, 7). Several nested PCR assays that used an initial set of primers to amplify both ST types and then amplification of an aliquot of the first reaction mixture with primer sets specific to either ST_h or ST_p have been used to type clinical ETEC isolates (5, 9, 17). Nested PCR assays are very sensitive; however, they are subject to contamination with carryover amplicon. Thus, we chose to incorporate the dUTP–uracil-*N*-glycosylase carryover contamination control system (11) and to use oligonucleotide hybridization probes to type ST alleles. All three ST probes (RDR250, RDR283, and RDR387) successfully combined to detect representatives of the types of ST, and they can be used independently for epidemiologic purposes. Probe hybridization assays to detect ETEC isolates that relied on single oligonucleotides were insensitive and demonstrated cross-reactivities with other enteric bacteria, even when the oligonucleotides were similar to those used in the present study (13).

The multiplex PCR assay allowed for the amplification of gene sequences from all types of ETEC in a single tube, eliminating the effect of sampling that can occur when separate reactions for the detection of LT-producing and ST-producing ETEC are required. Modification of the LT-Pr2, ST-Pr1, and ST-Pr2 primers of Frankel et al. (7) was performed to increase the amplification efficiency when the primer sets were combined in the multiplex reaction, as well as to eliminate the nonspecific amplification products produced by the LT primer set and to include ST_p-containing ETEC.

Purification of nucleic acids through binding to silica or glass particles in the presence of chaotropic agents and then elution in low-ionic-strength buffer is a widely known procedure. A simple and rapid protocol was developed by Boom et al. (3). In that protocol nucleic acids (various forms of DNA and RNA) were directly purified from human serum and urine by using guanidine thiocyanate and silica particles. However, they did not evaluate the performance of the protocol using clinical specimens containing cells (bacterial, fungal, or mammalian) or virus particles. Application to clinical specimens depends on efficient lysis of the pathogen and stabilization of the released DNA or RNA for subsequent purification on the silica or glass particles and amplification. They did find that size-fractionated silica particles were inadequate for purification of nucleic acids from cell-rich sources, such as suspensions of gram-negative bacteria, while diatoms performed adequately. The procedure was found to be inadequate for lysis of gram-positive bacteria or fungi. A study of the detection of enteroviruses in stool specimens successfully used this procedure; however, few details were supplied (25). In the present study, we found that a guanidine thiocyanate-containing buffer similar in composition to that used by Boom et al. (3) readily lysed the ETEC cells present in stool specimens obtained from infected patients and that purification of the nucleic acids in the presence of the chaotropic solution with a commercially available glass matrix resulted in a simple and rapid technique for preparing extracts relatively free of inhibitors to PCR but maintaining a high level of recovery of the target DNA. Stool specimens that were extremely variable in composition and state were all usable for PCR analysis when this procedure was used. This protocol for preparing stool specimens for PCR analysis has been successfully used for specimens containing *Salmonella* and *Campylobacter* spp., *Giardia lamblia*, and *Mycobacterium avium* (20) (data not shown). A 1:100 dilution of extracted DNA from either formed or liquid specimens was tested, and only the negative specimens were analyzed for the presence of inhibitors to PCR by the addition of exogenous control target DNA. The 1:100 dilution was rarely inhibitory to PCR. If the 1:100 dilution was not inhibitory, then a 1:50 dilution was assayed for target DNA.

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