JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1997, p. 281–283 Vol. 35, No. 1
0095-1137/97/$04.00+0

Rapid and Effective Method for Preparation of Fecal Specimens for PCR Assays

QINYUAN LOU,[1] SONNY K. F. CHONG,[1] JOSEPH F. FITZGERALD,[1]
JEAN A. SIDERS,[2] STEPHEN D. ALLEN,[2] AND CHAO-HUNG LEE[2]*

Departments of Pediatrics[1] and Pathology and Laboratory Medicine,[2]
Indiana University School of Medicine, Indianapolis, Indiana 46202

Received 28 June 1996/Returned for modification 30 August 1996/Accepted 3 October 1996

We have developed a novel method for the preparation of fecal specimens for PCR assays. Approximately 100 mg of solid stool or 200 μl of liquid fecal sample was thoroughly suspended in 1 ml of water. Fecal debris was removed by low-speed centrifugation (2,800 × *g* for 2 min). The supernatant was then boiled for 10 min in a water bath and further clarified by high-speed centrifugation (12,000 × *g* for 5 min). Fifty microliters of the clarified supernatant was then purified by Sepharose CL-6B spin column chromatography, and a portion of the purified supernatant was used for PCR. By this method, stools containing enterotoxigenic *Escherichia coli* H10407 were amplified by colonization factor antigen I fimbrial gene PCR, with a sensitivity of 100 organisms per reaction. The method was also effective for processing stool specimens for *Clostridium difficile* toxin A and B gene PCRs. This method is rapid, effective, and simple to perform and will improve the applications of PCR to stool specimens for diagnostic purposes.

PCR is a powerful molecular biology technique for the detection of target DNA in various clinical specimens, but its application to fecal specimens has been very limited because of the presence of unknown PCR inhibitors in such specimens. Many methods have been described for removal or inactivation of PCR inhibitors in fecal specimens. The conventional phenol-chloroform extraction method appears to be the most commonly used (2, 6, 13, 14, 19–21). Treatment with a high concentration of NaOH followed by extraction with phenol-chloroform has also been reported (2). Small-scale column chromatography with Sephadex G-50, cellulose fiber powder, or a glass matrix (19, 20) and a simple heating, washing, and dilution of specimens have also been described (2, 9). These methods are either laborious or inefficient. Various commercially available kits have also been used to prepare fecal specimens for PCR (4, 6, 7, 10, 13, 14, 17, 19–21). In this paper, we describe the use of Sepharose CL-6B spin column chromatography to process stool specimens for PCR.

Initially, three stool specimens from healthy volunteers, each spiked with 10,000 CFU of enterotoxigenic *Escherichia coli* (ETEC) H10407, were used to develop the purification techniques. Approximately 100 mg of solid stool or 200 μl of liquid fecal sample was suspended in 1 ml of sterile water. The suspension was centrifuged at 2,800 × *g* for 2 min to pellet larger fecal debris. Five hundred microliters of the supernatant was transferred to a new tube, boiled in a water bath for 5 min to lyse bacteria, and clarified by centrifugation at 12,000 × *g* for 5 min. The clarified supernatant was extracted with phenol-chloroform, and the DNA in the aqueous phase of the extraction was precipitated with ethanol as described previously (15). The DNA precipitates were pelleted by centrifugation and then washed with 70% ethanol. After removal of the ethanol by vacuum drying, the DNA was dissolved in 50 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). Ten microliters of the DNA solution was used as a template for PCR. The primers *cfaB*-1 (GCGGCTCTAATTAGATCT) and *cfcB*-p4 (GTG GTCAGACCATTGCACC) were used to amplify a 370-bp fragment of the *cfaB* gene of the colonization factor antigen I operon, which encodes the colonization factor antigen I fimbriae of ETEC (11). The 370-bp PCR product was generated from stools that were spiked with ETEC H10407 but not from stools containing no ETEC H10407. This result indicates that the *cfaB* PCR is specific for ETEC H10407.

Since it is quite laborious to process stool specimens for PCR by the conventional organic extraction method, the feasibility of using spin column chromatography to process stool specimens for PCR was explored. Three different chromatography matrices (Sepharose CL-6B, Sephadex G-50, and Sephadex G-25) were used. Stool specimens were processed by spin column chromatography for PCR as follows. After the 5-min centrifugation at 12,000 × *g* described above, 10 μl of the clarified supernatant was mixed with 30 μl of sterile water and 10 μl of a 5× stop solution (25% glycerol, 2% sodium dodecyl sulfate, 0.05% bromophenol blue, 0.05% xylene cyanol). The sodium dodecyl sulfate in the stop solution will dissociate proteins from DNA, and the bromophenol blue and xylene cyanol dyes serve as indicators for the effectiveness of the spin column chromatography. The mixture was heated in a 70°C water bath for 5 min and then purified by spin column chromatography with Sepharose CL-6B, Sephadex G-50, or Sephadex G-25. Preparations of these gel chromatography matrices and spun columns were as described previously (15). The same three stool specimens with or without spiked ETEC H10407 were examined. Only the stool sample containing ETEC H10407 processed by Sepharose CL-6B spin column chromatography produced positive PCR results.

Experiments were then performed to determine the efficiency of purification by Sepharose CL-6B spin column chromatography. Aliquots of approximately 100 mg each of a stool specimen were spiked with various numbers (10^4, 10^3, 10^2, 10^1, and 10^0 CFU) of ETEC H10407 organisms and processed for PCR by Sepharose CL-6B spin column chromatography. The same numbers of ETEC H10407 organisms were suspended in 40 μl of sterile water and then mixed with 10 μl of stop mixture, boiled in a water bath for 5 min, and purified by

* Corresponding author. Mailing address: Department of Pathology & Laboratory Medicine, Indiana University School of Medicine, 1120 South Dr., FH 419, Indianapolis, IN 46202. Phone: (317) 274-2596. Fax: (317) 278-0643. E-mail: chlee@indyvax.iupui.edu.

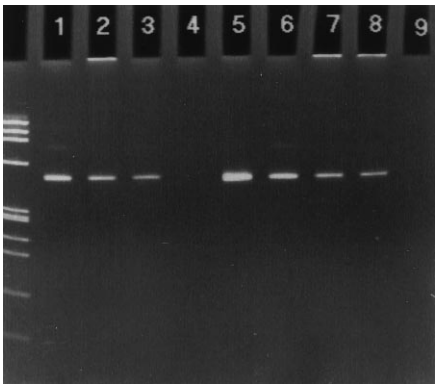

FIG. 1. Determination of efficiency of Sepharose CL-6B spin column chromatography for processing stool specimens for PCR. Aliquots of a stool specimen were spiked with various numbers of ETEC H10407 organisms and processed for PCR by Sepharose CL-6B spin column chromatography (lanes 1 to 4). The same numbers of ETEC H10407 organisms were suspended in 40 μl of sterile water and then mixed with 10 μl of stop mixture, boiled in a water bath for 5 min, and processed by Sepharose CL-6B spin column chromatography as controls (lanes 5 to 9). The numbers of CFU in each reaction mixture of spiked stool samples are as follows: lane 1, 10,000; lane 2, 1,000; lane 3, 100; and lane 4, 10. The numbers of CFU in each reaction mixture of control samples are as follows: lane 5, 10,000; lane 6, 1,000; lane 7, 100; lane 8, 10; and lane 9, 1. *Hae*III-digested φX174 DNA is shown as a size marker.

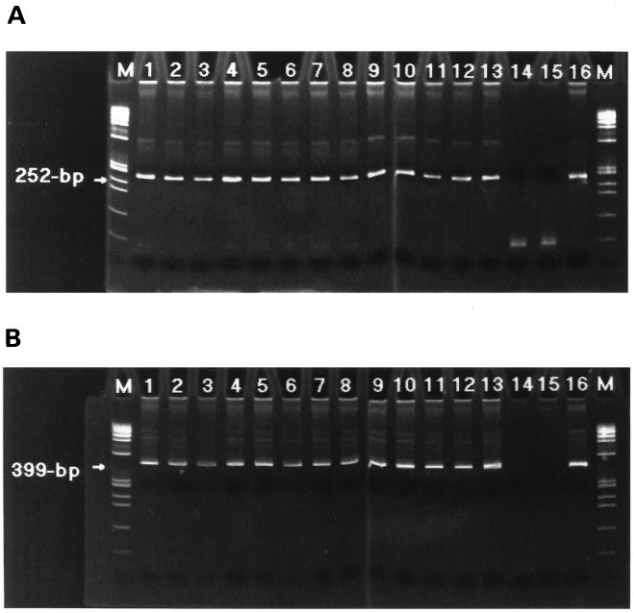

FIG. 2. Results of *C. difficile* toxin A (A) and B (B) gene PCRs. Thirteen *C. difficile* culture-positive stools and two *C. difficile* cultures were examined by the toxin A and toxin B gene PCRs. PCR products from each sample were electrophoresed on a 6% polyacrylamide gel in different lanes. Lanes 1 to 13 contain PCR products of the 13 stool samples. Lane 14 contains PCR products of the negative control. Lanes 15 and 16 contain PCR products of the cytotoxin-negative and cytotoxin-positive *C. difficile* cultures, respectively. The toxin A gene PCR product is 252 bp and that of the toxin B gene PCR is 399 bp, as indicated by arrows. *Hae*III-digested φX174 DNA (M) was used as size markers.

Sepharose CL-6B spin column chromatography as controls. Ten microliters of each purified sample was used for PCR. The stool samples containing 10^2 CFU or more ETEC H10407 organisms and control samples containing 10^1 or more ETEC H10407 organisms gave positive PCR results (Fig. 1). These results indicate that Sepharose CL-6B spin column chromatography can remove most PCR inhibitors present in stool specimens.

The method was then applied to 20 stool specimens to detect the *C. difficile* toxin A and B genes by PCR. These specimens were obtained from the Clinical Microbiology Laboratory, Indiana University Medical Center. Thirteen of them were positive by *C. difficile* culture. The *C. difficile* isolates from these 13 specimens were all positive by the cytotoxicity assay, suggesting the presence of the toxin B gene. A cytotoxin-positive culture and a cytotoxin-negative *C. difficile* culture were used as controls. *C. difficile* cultures were performed by the direct plating method (16) using cycloserine-cefoxitin-fructose agar with horse blood (Carr-Scarborough Microbiologicals, Inc., Stone Mountain, Ga.). Identifications of *C. difficile* isolates were made by using conventional media and methods (3, 8). Cytotoxin assays were done with the Techlab (Blacksburg, Va.) *C. difficile* Tox-B test.

Approximately 10,000 CFU of each *C. difficile* culture was suspended in 1 ml of water, and then the suspensions were processed as if they were stool specimens for PCR. Both toxin A and B gene PCRs were performed. Two sets of primers were used to detect *C. difficile* in clinical specimens. NK2 (CCCAA TAGAAGATTCAATATTAAGCTT) and NK3 (GGAAGAA AAGAACTTCTGGCTCACTCAGGT) were used to amplify a 252-bp fragment of the toxin A gene (12, 13), while YT-17 (GGTGGAGCTTCAATTGGAGAG) and YT-18 (GTGTAA CCTACTTTCATAACACCAG) were used to amplify a 399-bp fragment of the toxin B gene (7). PCRs were performed in a 100-μl reaction mixture containing 10 μl of template DNA mentioned above, PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 2 mM $MgCl_2$; 0.001% gelatin), 0.2 μM each PCR primer, 0.2 mM each deoxynucleoside triphosphate, and 2 U of *Taq* DNA polymerase. After a 10-min denaturation at 94°C, the PCR mixtures were subjected to 30 cycles of amplification at 94°C for 2 min, 55°C for 30 s, and 72°C for 1 min. The PCR products were electrophoresed on a 6% polyacrylamide gel to determine their sizes.

The cytotoxin-positive *C. difficile* culture was positive by both the toxin A and B gene PCRs, whereas the cytotoxin-negative *C. difficile* culture was negative by both the toxin A and B gene PCRs (Fig. 2 and 3). These results indicated that both the toxin A and B gene PCRs worked properly. The 20 stool specimens were then assayed by both the toxin A and B gene PCRs. The 13 *C. difficile* culture-positive stools were found to be positive by both the toxin A and B gene PCRs (Fig. 2 and 3), and the 7 *C. difficile* culture-negative stools were negative. These *C.*

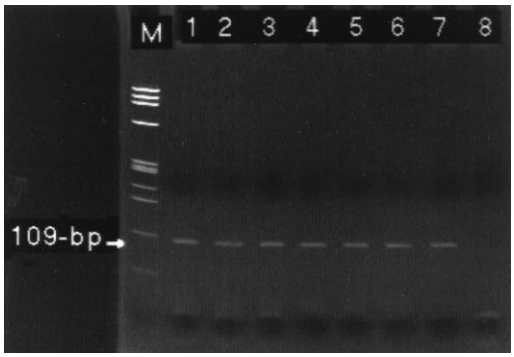

FIG. 3. Results of PCR of seven *C. difficile* culture-negative stool samples with Hp1 and Hp2. Lanes 1 to 7 contain PCR products (109 bp) from the seven stool specimens. Lane 8 contains PCR products of the negative control. *Hae*III-digested φX174 DNA (M) was used as size markers.

difficile PCR-negative stool specimens were further examined with primers Hp1 and Hp2, which have been shown to amplify a 109-bp fragment from the human genome (5). Positive PCR results were generated, indicating that the DNA from these seven specimens was amplifiable and that the negative *C. difficile* PCR results were not due to the presence of inhibitors in the specimens.

Processing of specimens is the most critical step in PCR assays. Although it is not essential to isolate DNA in great purity, it is necessary to remove or inactivate substances that may inhibit PCR assays. Blood and stool specimens are known to contain potent PCR inhibitors, most of which have not been identified. The method described in this paper appears to be very effective and simple. A small amount of a stool sample is first suspended in an ample amount of water to decrease the concentration of PCR inhibitors. The fecal debris, which is inhibitory to PCR, is then removed by low-speed centrifugation. The clarified supernatant is boiled to lyse bacteria and release bacterial DNA. The proteins and other substances in the solution are separated from DNA by spin column chromatography. Sephadex G-25, Sephadex G-50, and Sepharose CL-6B, with exclusion limits of 1,000 to 5,000, 1,500 to 30,000, and 10,000 to 4,000,000 Da, respectively, were used. Only Sepharose CL-6B achieved a satisfactory effect, suggesting that the molecular masses of inhibitors may range from 30,000 to 4,000,000 Da.

Many methods for processing stool specimens for PCR have been described. The steps common among these methods are suspending stool samples in solutions and then pelleting larger fecal debris by low-speed centrifugation. The clarified supernatant is then subjected to high-speed centrifugation, after which either the pellet (1, 13, 14, 18, 19) or the supernatant (2, 4, 17, 21) is saved for PCR analysis. In this study, we were unable to obtain positive PCR results with the pellet. A possible reason for this failure is that the pellet still contained significant amounts of fecal debris which could not be effectively removed by subsequent purification processes. We overcame this problem by lysing bacteria after the low-speed centrifugation step, followed by high-speed centrifugation to pellet all debris. The highly clarified supernatant was then used for PCR analyses. We also found that the solutions for suspending stool samples did not appear to be critical since we have obtained the same results with sterile water or TE buffer.

Phenol-chloroform treatment followed by ethanol precipitation is the classical method for preparation of stool specimens for PCR assay. Phenol-chloroform extraction eliminates biological contaminants in stool suspensions that may inhibit PCR, but this method appears to be unable to remove nonbiological substances (13, 19, 20). Wilde et al. reported that phenol-chloroform extraction failed to remove the inhibitors unless the specimens were further purified with chromatography medium CF11 (20). The alkali method for inactivation of PCR inhibitors in stool specimens must be followed by addition of HCl to neutralize the solution, which increases the concentrations of salt and ions that are also inhibitory to PCR. The method developed in our study does not require phenol-chloroform extraction, proteinase K digestion, or ethanol precipitation. It removes PCR inhibitors in stool specimens by using Sepharose CL-6B spin column chromatography. This method is rapid, effective, and inexpensive and is theoretically applicable to any type of specimen.

REFERENCES

1. **Acuna-Soto, R., J. Samuelson, P. D. Girolami, L. Zarate, F. Millan-Velasco, G. Schoolnick, and D. Wirth.** 1993. Application of the polymerase chain reaction to the epidemiology of pathogenic and nonpathogenic *Entamoeba histolytica*. Am. J. Trop. Med. Hyg. **48:**58–70.
2. **Allard, A., R. Girones, P. Juto, and G. Wadell.** 1990. Polymerase chain reaction for detection of adenoviruses in stool samples. J. Clin. Microbiol. **28:**2659–2667.
3. **Allen, S. D., and E. J. Baron.** 1991. *Clostridium*, p. 505–521. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
4. **Boondeekhun, H. S., V. Gurtler, M. L. Odd, V. A. Wilson, and B. C. Mayall.** 1993. Detection of *Clostridium difficile* enterotoxin gene in clinical specimens by the polymerase reaction. J. Med. Microbiol. **38:**384–387.
5. **Chong, S. K. F., Q. Lou, J. F. Fitzgerald, and C.-H. Lee.** 1996. Evaluation of 16S rRNA gene PCR with primers Hp1 and Hp2 for detection of *Helicobacter pylori*. J. Clin. Microbiol. **34:**2728–2730.
6. **Gumerlock, P. H., Y. J. Tang, F. J. Meyers, and J. Silva, Jr.** 1991. Use of the polymerase chain reaction for the specific and direct detection of *Clostridium difficile* in human feces. Rev. Infect. Dis. **13:**1053–1060.
7. **Gumerlock, P. H., Y. J. Tang, J. B. Weiss, and J. Silva, Jr.** 1993. Specific detection of toxigenic strains of *Clostridium difficile* in stool specimens. J. Clin. Microbiol. **31:**507–511.
8. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
9. **Hornes, E., Y. Wasteson, and Ø. Olsvik.** 1991. Detection of *Escherichia coli* heat-stable enterotoxin genes in pig stool specimens by an immobilized, colorimetric, nested polymerase chain reaction. J. Clin. Microbiol. **29:**2375–2379.
10. **Ibrahim, A., W. Liesack, and E. Stackebrandt.** 1992. Polymerase chain reaction-gene probe detection system specific for pathogenic strains of *Yersinia enterocolitica*. J. Clin. Microbiol. **30:**1942–1947.
11. **Karjalainen, T. K., D. G. Evans, M. So, and C.-H. Lee.** 1989. Molecular cloning and nucleotide sequence of the colonization factor antigen I gene of *Escherichia coli*. Infect. Immun. **57:**1126–1130.
12. **Kato, N., C.-Y. Ou, H. Kato, S. L. Bartley, V. K. Brown, V. R. Dowell, Jr., and K. Ueno.** 1991. Identification of toxigenic *Clostridium difficile* by the polymerase chain reaction. J. Clin. Microbiol. **29:**33–37.
13. **Kato, N., C. Y. Ou, H. Kato, S. L. Bartley, C. C. Luo, G. E. Killgore, and K. Ueno.** 1993. Detection of toxigenic *Clostridium difficile* in stool specimens by the polymerase chain reaction. J. Infect. Dis. **167:**455–458.
14. **Kuhl, S. J., Y. J. Tang, L. Navarro, P. H. Gumerlock, and J. Silva, Jr.** 1993. Diagnosis and monitoring of *Clostridium difficile* infections with the polymerase chain reaction. Clin. Infect. Dis. **16**(Suppl. 4)**:**S234–S238.
15. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Marler, L. M., J. A. Siders, L. C. Wolters, Y. Pettigrew, B. L. Skitt, and S. D. Allen.** 1992. Comparison of five cultural procedures for isolation of *Clostridium difficile* from stools. J. Clin. Microbiol. **30:**514–516.
17. **Olive, D. M.** 1989. Detection of enterotoxigenic *Escherichia coli* after polymerase chain reaction amplification with a thermostable DNA polymerase. J. Clin. Microbiol. **27:**261–265.
18. **Saulnier, P., and A. Andremont.** 1992. Detection of genes in feces by booster polymerase chain reaction. J. Clin. Microbiol. **30:**2080–2083.
19. **Stacy-Phipps, S., J. J. Mecca, and J. B. Weiss.** 1995. Multiplex PCR assay and simple preparation method for stool specimens detect enterotoxigenic *Escherichia coli* DNA during course of infection. J. Clin. Microbiol. **33:**1054–1059.
20. **Wilde, J., J. Eiden, and R. Yolken.** 1990. Removal of inhibitory substances from human fecal specimens for detection of group A rotaviruses by reverse transcriptase and polymerase chain reactions. J. Clin. Microbiol. **28:**1300–1307.
21. **Xu, L., D. Harbour, and M. A. McCrae.** 1990. The application of polymerase chain reaction to the detection of rotaviruses in feces. J. Virol. Methods **27:**29–38.