

Specific Detection of Toxigenic Strains of *Clostridium difficile* in Stool Specimens

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Clostridium difficile is the infectious agent responsible for antibiotic-associated colitis. We report the use of the polymerase chain reaction technique to identify toxigenic strains of *C. difficile* in human stool specimens. A set of primers based on the nucleotide sequence of the toxin B gene, which amplified a 399-bp fragment from isolates producing toxin B, was designed. We examined 28 known toxigenic strains, which were all positive by this assay. DNAs from the nontoxigenic strains examined and from strains of *Clostridium sordellii* and *C. bifermentans* were not amplified with these primers. The sensitivity of this assay allowed us to identify as little as 10% toxigenic *C. difficile* cells in the presence of 90% nontoxigenic cells and to detect the toxin B gene in 1 pg of DNA from a toxigenic strain. DNAs extracted from 18 clinical stool specimens that were positive for toxin B by the tissue culture cytotoxicity assay were also positive by this assay. In addition, we detected toxin B sequences in DNA from 2 of 18 stool specimens that were negative for toxin B by the cytotoxicity assay. These two stool specimens were from patients who had a clinical pattern of colitis that was compatible with *C. difficile* causation. This rapid, sensitive assay will be useful for specific identification of toxigenic *C. difficile* and for revealing cases that are undetected by analysis of fecal samples for toxin B alone.

Clostridium difficile is the etiologic agent of pseudomembranous colitis and antibiotic-associated colitis (10). This anaerobic bacterium produces at least two toxins: toxin A, an enterotoxin, and toxin B, a potent cytotoxin (7). These toxins act synergistically to cause extensive tissue damage and fluid accumulation, which result in patient symptoms and disease.

Diagnosis of *C. difficile*-induced colitis is made by testing for the presence of toxins A and B in fecal specimens. The toxin B assay is a fibroblastic tissue culture assay in which cytopathologic effects are observed after the addition of stool filtrates rendered free of bacteria to the cultured cells. Many hospitals lack the facilities to perform this assay; in addition, it has a 5% false-negative rate because of toxin degradation by naturally occurring proteases present in feces or instability with storage (freezing and thawing). Clinical tests specific for toxin A include a dot immunobinding assay and several enzyme immunoassays. These tests, although simple to perform, do not detect strains of *C. difficile* that produce low levels of toxin and thus occasionally give indeterminate results.

Recently, the toxin B gene from *C. difficile* was cloned and sequenced (1, 8, 17). This has allowed the application of the polymerase chain reaction (PCR) technique for the detection of toxigenic strains of *C. difficile* (19). Others have developed PCR-based assays to detect toxin A sequences in strains of *C. difficile* (9, 20). These PCR-based toxin studies have only been performed on DNA from *C. difficile* grown in cultures and have not been applied to clinical specimens. However, the PCR was previously used by us to detect the rRNA gene of *C. difficile* (toxigenic and nontoxigenic strains) directly in human fecal samples (6).

In this study, we report the use of PCR to identify toxigenic strains of *C. difficile* directly in the stool of patients. The specificity and sensitivity of our PCR tech-

nique in detecting toxigenic strains of *C. difficile* in a mixed population with nontoxigenic strains were determined. This assay may be useful in rapidly identifying infected patients and can contribute to more timely therapeutic intervention for patients.

MATERIALS AND METHODS

Bacterial strains and culture media. The *C. difficile* strains used in this study were isolates from patients at the University of California, Davis Medical Center (28 toxigenic strains and 22 nontoxigenic strains). The toxigenic strains showed various degrees of toxigenicity, including weak toxigenicity. *Clostridium sordellii* (Centers for Disease Control), *C. bifermentans* (ATCC 638), and *Escherichia coli* (University of California, Davis Medical Center) were also used. *C. difficile* was cultured from stool specimens on plates of cycloserine-cefoxitin-fructose-agar, a medium highly selective for *C. difficile* (5). All medium was reduced for 5 h before use. Cultures were grown anaerobically at 37°C for 48 h. After purification, *C. difficile* was identified by a rapid latex agglutination test (Serobact Disposable Products Pty. Ltd., Adelaide, South Australia), Gram staining, and a toxin B cytotoxicity assay. All *Clostridium* cultures were maintained in cooked meat broth (Central Media, Sacramento, Calif.).

Toxin B cytotoxicity assay. Cytotoxicity assays for toxin B were performed as described elsewhere (16). Fresh specimens were used in all cases. Briefly, 96-well flat-bottom sterile microtiter plates were seeded with 100 µl of human embryonic lung cells (MA Bioproducts, Walkersville, Md.) and inoculated with 100 µl of stool filtrate (undiluted and serially 10-fold diluted to 10⁻³) or with a culture filtrate (undiluted and serially diluted 10-fold to 10⁻⁴). These same dilutions of stool or culture filtrates were also mixed with an equal volume of a 1:20 dilution of the stock solution of *C. difficile* antitoxin (VPI Anaerobe Laboratories, Blacksburg, Va.) and incubated at 37°C for 10 min, and then 100 µl was added to the cells in the microtiter plates. Cell culture

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medium, antitoxin, and toxin controls were included in each run. The plates were incubated at 37°C in 7% CO₂ for 24 h, examined, and scored for cytopathic effects. They were incubated for an additional 48 h and again scored for cytopathic effects.

Extraction of DNA from bacterial isolates. DNA extraction from *Clostridium* spp. was accomplished by growing the bacteria in 50 ml of prereduced brain heart infusion broth anaerobically at 37°C for 48 h. Cells were harvested by centrifugation at 6,000 × *g* for 20 min and washed once in 50 mM Tris (pH 7.4). The pellet was suspended in 2 ml of Tris-borate-EDTA (TBE) buffer (pH 8). Lysozyme was added to a final concentration of 0.5 mg/ml, the suspension was incubated at 37°C for 15 min, and then 20% sodium dodecyl sulfate (SDS) was added to a final concentration of 0.5%. After 10 min of incubation at 60°C, the mixture was centrifuged at 1,000 × *g* for 10 min to remove broken cells and debris. The supernatant was extracted several times with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) until a clear interface was obtained. The DNA was precipitated with 2 volumes of ice-cold ethanol. The DNA pellet was dried and redissolved in Tris-EDTA buffer (pH 7.4). DNA concentration was determined spectrophotometrically by reading the *A*₂₆₀, setting 1 *A*₂₆₀ unit equal to 50 µg/ml.

Extraction of DNA from stools. DNA was extracted from stool specimens by using EXTRACTOR-15 columns as previously described (6). Both fresh and frozen specimens were used.

DNA amplification from bacteria and stool. The sequences of the oligonucleotides used as primers and the probe in this study are as follows: downstream primer (YT-17), GGTG GAGCTTCAATTGGAGAG; upstream primer (YT-18), GT GTAACCTACTTTCATAACACCAG; probe (YT-20), GTG GAGTGTTACAAACAGGTG. The PCR method of Mullis and Faloona was used for amplification with the thermostable DNA polymerase (*Taq*; Perkin Elmer-Cetus) (14). The reaction mixtures were prepared in 1× PCR buffer (50 mM KCl, 20 mM Tris-HCl, 2.5 mM MgCl₂, 100 µg of bovine serum albumin per ml [pH 8.4]) and contained (per reaction) 20 pmol of each of the primers, 0.1 mM (each) deoxynucleoside triphosphates, 2 U of *Taq* polymerase, and bacterial DNA at concentrations of 100 to 250 ng.

For stool specimens, 200 to 500 ng of DNA was routinely used, and the reaction mixture was supplemented with 5% glycerol. The use of glycerol when analyzing the stool specimens increased the sensitivity of detection. Reaction mixtures were covered with 150 µl of light mineral oil (Sigma Chemical Co., St. Louis, Mo.) to prevent evaporation. The PCR profile used throughout these experiments included a denaturing step at 95°C for 30 s followed by annealing of the primers at 55°C for 30 s with an extension for 30 s at 72°C. All amplifications were done for 40 cycles in a DNA thermal cycler (Perkin Elmer-Cetus). In all cases, a negative control containing all PCR reagents but no DNA was used to monitor for contamination. As a control for the quality of amplifiable clostridial DNA from stool specimens, DNA was first amplified with primers directed at the 16S rRNA gene as described previously (6). Only stool specimens positive for amplification with that pair of primers were used in the toxin B PCR assay.

Detection of amplified products. Initially, amplification products were visualized by running 9 µl of the reaction mixture in 2% NuSieve (FMC, Rockland, Maine)-1% agarose gels in TBE buffer. Size markers included in all gels were the 123-bp DNA ladder (BRL, Grand Island, N.Y.). Gels

were run in TBE at a constant 110 V for 90 min. Gels were stained in an ethidium bromide solution (0.5 µg/ml) for 30 min, destained for 30 min, and photographed under UV light with a Polaroid Land camera.

Southern blot analysis. A nonradioactive method employing horseradish peroxidase, a biotin-labeled oligomer, and an enzyme chemiluminescence detection system (Amersham, Arlington Heights, Ill.) was used as previously described (6). Hybridization of the blot was done with 10 pmol of probe YT-20 for 45 min at 45°C in 5× SSPE (1× SSPE is 150 mM NaCl-10 mM NaH₂PO₄ · H₂O-50 mM EDTA [pH 7.4])-0.5% SDS. Blots were washed for 10 min at room temperature in 2× SSPE-0.1% SDS and then for 20 min at 45°C in 5× SSPE-0.5% SDS. The blots were then incubated with streptavidin-horseradish peroxidase, and the blot was washed and developed as described previously (6). The blots were exposed to Kodak XAR film for 30 min to 2 h. The film was developed, and the results were interpreted.

RESULTS

Amplification of toxigenic strains of *C. difficile*. A pair of primers directed at the toxin B gene was used to specifically amplify toxin B sequences from strains of *C. difficile* by the PCR. With these primers, a 399-bp amplicon was obtained. Twenty-eight toxigenic strains and 22 nontoxigenic strains of *C. difficile* were tested. We amplified the expected 399-bp fragment in DNA from all the toxigenic *C. difficile* strains. No amplification products were observed with DNA from nontoxigenic strains (Fig. 1A). To confirm the specificity of the product, Southern blot analysis was done with a biotin-labeled internal probe, YT-20. As seen in Fig. 1B, only DNA products from toxigenic strains hybridized with the probe. None of the nontoxigenic strains probed positive.

Specificity of the PCR assay. To further determine the specificity of the PCR assay, amplification reactions were carried out with DNA from four strains of *C. sordellii* and one strain of *C. bifermentans*. These species were selected because they are closely related to *C. difficile* and because it is well documented that the hemorrhagic toxin protein of *C. sordellii* cross-reacts with toxin A of *C. difficile* in antibody-based assays (11). Some PCR-based techniques have also resulted in cross-reactivity between these two species (6, 20). No amplification products were obtained with DNAs from the strains of *C. sordellii* tested in this study (Fig. 2A), and these samples remained negative when probed with the internal probe (Fig. 2B).

Sensitivity of toxin B PCR assay. Before the sensitivity of our PCR assay was determined, experiments were done to determine the optimal MgCl₂ concentration for the primer pair YT-17 and YT-18. For this purpose, our PCR buffer was supplemented with MgCl₂ concentrations ranging from 0.5 to 4 µM. Amplification reactions were set up with DNA amounts ranging from 10 to 100 ng. With the lower amounts of DNA, more efficient amplification was obtained with MgCl₂ concentrations of 2.5 µM. This concentration was then used in the sensitivity experiments.

The sensitivity of this system in detecting toxigenic strains of *C. difficile* was determined with two experiments. In the first experiment, DNA from *C. difficile* in amounts equal to those found in 10 to 10⁴ cells was mixed with *E. coli* DNA equal to the amount in 10⁶ cells. After 40 cycles of amplification, as few as 100 cells of *C. difficile* could be detected among 10⁶ cells of *E. coli* (Fig. 3). The second experiment was designed to detect the sensitivity of our technique in detecting toxigenic strains of *C. difficile* in a mixed popula-

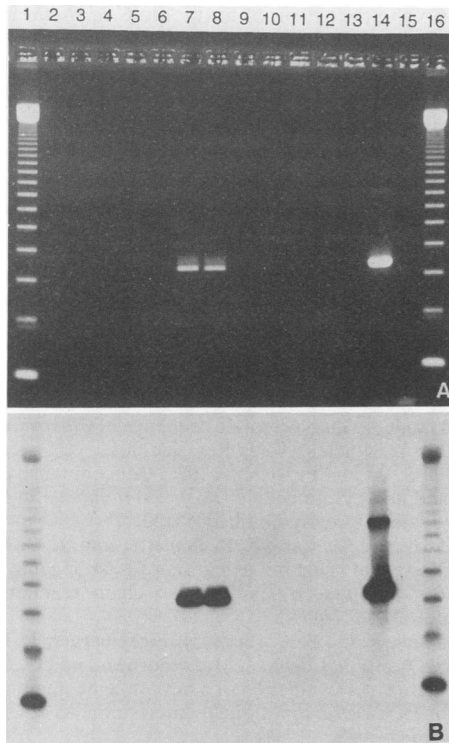


FIG. 1. Ethidium bromide-stained 2% agarose gel (A) and Southern blot (B) analysis of PCR products of DNA from toxigenic and nontoxigenic *C. difficile* amplified with primers directed at the toxin B gene. Lanes: 1 and 16, 123-bp DNA marker ladder; 4, 5, and 6, nontoxigenic strains; 7, 8, and 14, toxigenic strains; 13, *E. coli*; 15, negative control; 2, 3, 9, 10, 11, and 12, blank lanes.

tion with nontoxigenic strains. This would allow specific identification of toxigenic strains in patients colonized by more than one strain of *C. difficile*. For these experiments, we mixed DNA from a toxigenic strain in ratios that varied from 10 to 50% with DNA from a nontoxigenic strain in ratios of 90 to 50%, respectively. In a mixed population, as little as 10% toxigenic bacteria could be detected by our technique (data not shown).

Amplification of clinical specimens. Extracting DNA from stool specimens results in much variability from specimen to specimen in the yield and purity of DNA obtained. In addition, it has been reported that clinical specimens (stool and urine) contain inhibitors of DNA polymerase (18). As a control for amplifiable clostridial DNA extracted from stool, the DNA was amplified with the 16S rRNA primers B and PG-48 (6). Only specimens from which DNA amplification was obtained with the rRNA primers were screened with the toxin B PCR assay. Approximately 90% of the symptomatic patient specimens we studied were positive for the clostridial 16S rRNA amplicon. All 18 specimens that tested negative in the toxin B PCR assay tested negative in the cytotoxicity assay. All specimens that were positive for toxin B by the tissue culture assay were also positive by the PCR; the data for some of these are shown in Fig. 4. Toxin B gene amplification products were also produced from DNA extracted from two stool specimens of symptomatic patients which were reported negative by the cytotoxicity assay. These patients had a clinical pattern of colitis compatible with *C. difficile* causation, had been on antibiotic therapy for

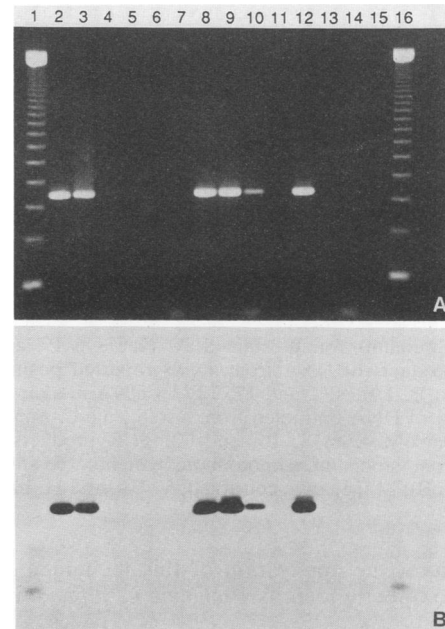


FIG. 2. Ethidium bromide-stained 2% agarose gel (A) and Southern blot (B) analysis of PCR products of DNA from *Clostridium* species. Lanes: 1 and 16, 123-bp DNA marker ladder; 2, 3, 8, 9, 10, and 12, toxigenic *C. difficile* strains isolated from patients; 4, *C. bifementans* (ATCC 638); 6 and 7, *C. sordellii*; 14, negative control; 5, 11, 13, and 15, blank lanes.

several days, had previous histories of *C. difficile* relapses for periods of 3 to 9 months, and had all of the symptoms characteristic of antibiotic-associated colitis.

DISCUSSION

This report outlines the use of PCR to identify toxigenic strains of *C. difficile* by amplification of toxin B gene sequences directly from the stools of human patients. The PCR has been used previously by several investigators to identify toxigenic strains of *C. difficile* (12, 13, 19, 20). In all of these studies, amplification reactions were only performed on DNA extracted from strains of *C. difficile* grown

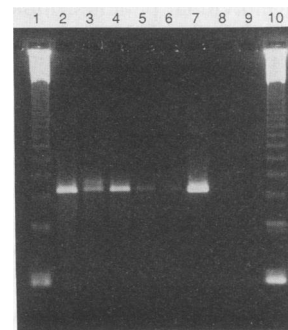


FIG. 3. Sensitivity of the PCR technique in detecting toxigenic *C. difficile* DNA contained in 10^2 to 10^6 cells in the presence of *E. coli* DNA (10^6 cells). Lanes: 1 and 10, 123-bp DNA marker ladder; 2 through 6, 10^6 , 10^5 , 10^4 , 10^3 , and 10^2 cells of toxigenic *C. difficile*, respectively, with 10^6 cells of *E. coli*; 7, 10^7 cells of *C. difficile*; 8, negative control; 9, blank lane.



FIG. 4. Ethidium bromide-stained 2% NuSieve-1% agarose gel with PCR products of DNA from stools reported positive for *C. difficile* toxin B. Lanes: 1 and 17, 123-bp DNA marker ladder; 2, PCR product of DNA extracted from a toxigenic *C. difficile* strain (positive control); 4, 6, 8, 10, and 12, PCR products of DNA extracted from stools of symptomatic patients (positive for *C. difficile* toxin B); 14, negative control; 3, 5, 7, 9, and 11, blank lanes.

in vitro. Here, the application of this technique has been extended to directly identify toxigenic *C. difficile* in clinical specimens.

The current methodologies to diagnose *C. difficile* colitis have variable sensitivities and specificities. Some of the recent commercially available enzyme-linked immunosorbent assays have been shown to produce indeterminate results in symptomatic patient stool specimens (4). In this study, we investigated a number of toxigenic strains. In all cases, we were able to amplify the target 399-bp fragment in the DNA obtained from these strains. The sensitivity of our assay allowed the detection of toxin B sequences when only 1 pg of toxigenic bacterial DNA was present. This degree of sensitivity is useful, particularly when the assay is applied to clinical specimens. In our experience, results obtained in the tissue culture assay for toxin B are positive when at least 10^4 *C. difficile* organisms are present per g in the specimen. Thus, sensitivity obtained with our assay represents a 10- to 100-fold increase in the ability to detect toxigenic *C. difficile*. This degree of sensitivity is comparable to that reported by others using PCR in other systems (3, 15).

The sensitivity of our assay in detecting toxigenic strains of *C. difficile* in a mixed population with nontoxigenic strains was determined. This would be of importance for patients colonized by more than one strain of *C. difficile*. Toxigenic and nontoxigenic isolates have been cultured concurrently from the stools of individual patients, indicating that more than one strain of *C. difficile* can be harbored simultaneously (2). Conventional tests require the isolation and testing of a number of separate colonies obtained from the same stool specimen to determine whether more than one strain of *C. difficile* is present. Although the experiments presented in this study were done in vitro with different DNA concentrations from a toxigenic and a nontoxigenic strain, we speculate that the toxin B PCR assay in conjunction with the 16S rRNA PCR assay can be applied to identify colonization of an individual patient with toxigenic or nontoxigenic *C. difficile* or both (6). A ratio of the amount of amplification obtained with both systems (toxin B/rRNA) would be used. A semiquantitative approach to the load of *C. difficile* during the course of infection and treatment may provide some new understanding of the disease process of *C. difficile* colitis.

In summary, the toxin B PCR assay is a sensitive and specific method for the detection of toxigenic *C. difficile* in clinical specimens. The high degree of sensitivity of this

assay allows the detection of toxigenic *C. difficile* in cases in which toxin B is undetected by the conventional cytotoxicity assay. Thus, PCR-based assays may be very useful at the clinical level in the identification and prompt treatment of infected patients.

ACKNOWLEDGMENTS

We thank Janet Young for technical assistance. We also thank Gloria Marquez for excellent typing of this manuscript.

This work was supported in part by the State of California Competitive Technology Program (grant CT90B-075). P. H. Gumerlock was supported in part by the Mathews Foundation for Prostate Cancer Research.

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