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Development and Evaluation of a PCR Method for Detection of the *Clostridium difficile* Toxin B Gene in Stool Specimens

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A PCR assay detecting *Clostridium difficile* toxin B gene in stool specimens was compared to the cytotoxicity assay as the reference standard for the diagnosis of *C. difficile* antibiotic-associated diarrhea (CDAD). Overall, 118 stool samples were tested. All of the specimens that were negative by the cytotoxicity assay (59 out of 118) were also negative by the PCR method (specificity of 100%). Of the 59 cytotoxin-positive samples, 54 were PCR positive (sensitivity of 91.5%). This PCR method is promising for rapid diagnosis of CDAD.

Clostridium difficile is a frequent cause of antibiotic-associated diarrhea (CDAD) and colitis (14, 16). Pathogenic strains of *C. difficile* produce two toxins, A and B, that are involved in the pathogenicity of the organism (6, 16). Toxin A is an enterotoxin responsible for tissue damage while toxin B is referred to as a potent cytotoxin (13, 16). Until recently, it was thought that all toxigenic strains of *C. difficile* produced both toxins A and B, whereas nontoxigenic strains failed to produce the toxins and were not pathogenic (11, 16). However, *C. difficile* strains not producing detectable toxin A but still producing toxin B (7, 10, 15, 24) and retaining the ability to cause disease in humans (1, 21) have been identified. The genes coding for toxin A (*tcdA*) and toxin B (*tcdB*) are part of a 19.6-kb genetic locus (pathogenicity locus [PaLoc]) that includes three additional small open reading frames (*tcdC*, *tcdD*, and *tcdE*) (8, 12). Cohen et al. (9) have suggested that the PaLoc is highly stable in toxigenic *C. difficile* while nontoxigenic isolates were lacking the unit. A−/B+ strains of *C. difficile* (strain 1470 and strain 8864) are truncated at the 3′ ends of their toxin A genes (*tcdA*) (20, 22, 23).

Rapid identification of *C. difficile* is important for patient management and prompt epidemiological interventions. The reference standard for the laboratory diagnosis of CDAD is the cytotoxicity assay, which detects primarily toxin B (2). This method is highly sensitive (17) and correlates well with disease but is labor-intensive (2) and time-consuming and has a 48- to 72-h turnaround time. Because of the relative difficulty of extracting DNA from fecal specimens, few studies have been conducted using PCR to detect *C. difficile*. To increase the sensitivity of the assay, some investigators have developed a nested PCR approach (3, 4). Recently, a commercial extraction system, the QIAamp DNA Stool Mini Kit (QIAGEN, Mississauga, Ontario, Canada), became available. This kit is intended to provide fast and easy purification of total DNA from stool samples. Therefore, its use enabled us to develop a rapid and simple PCR assay for the detection of the *C. difficile* toxin B gene. We also evaluated the performance of the assay on clinical specimens in comparison to the cytotoxicity assay, and we determined the analytical sensitivity of the PCR assay.

Patients' stool specimens. Between 1 October 2000 and 18 February 2001, all stool specimens submitted for *C. difficile* toxin detection at the microbiology laboratory of Maisonneuve-Rosemont Hospital were routinely tested with the cytotoxicity assay. Fifty-nine consecutive stool samples positive by the cytotoxicity assay were selected for testing by PCR; for each cytotoxin-positive specimen, the following sequential stool specimen submitted to the laboratory for *C. difficile* detection and negative by the cytotoxicity assay was also selected for testing by PCR. Informed consent was obtained from the 118 patients whose specimens were selected for the study. All PCR testing was performed blinded to the results of the cytotoxicity assay. Stool pellets from the processing of the samples for the cytotoxicity assay were kept at −20°C and tested by PCR within 1 week of collection. All specimens were also cultured on a *C. difficile* selective medium (cycloserin, cefoxitin, and sheep blood agar; Quelab, Montreal, Canada).

Cytotoxicity assay. Specimens were treated with phosphate-buffered saline at 4°C for 24 h and then centrifuged. Filtrates of the supernatants were added to microtiter wells containing VERO cells (African green monkey kidneys) and processed with the *C. difficile* Toxin/Antitoxin Kit (TechLab, Blacksburg, Va.). The test was considered positive when cells showed a cytopathic rounding which was neutralized by specific *C. difficile* antitoxin. Positive results were determined after 24 and 48 h and negative results were determined after 48 h.

PCR assay. Total DNA was extracted from stool specimens by using the QIAamp DNA Stool Mini Kit per the manufacturer's instructions. Four microliters of each eluted sample was directly used for amplification. A 322-bp fragment was amplified with primers CDTB1 and CDTB2 derived from the nonrepeating portion of the *C. difficile* toxin B gene (3). PCRs were carried out in 50 μl of reaction volume and performed with *Taq* DNA Polymerase (QIAGEN) in a Perkin-Elmer 9600 thermal cycler. The amplification profile consisted of an initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 75 s, and a final extension at 72°C for 10 min. Amplicons were detected in a 1.4% agarose gel stained with ethidium bromide. Positive (pure DNA from toxigenic *C. difficile* strain ATCC 9689) and negative (pure DNA from a

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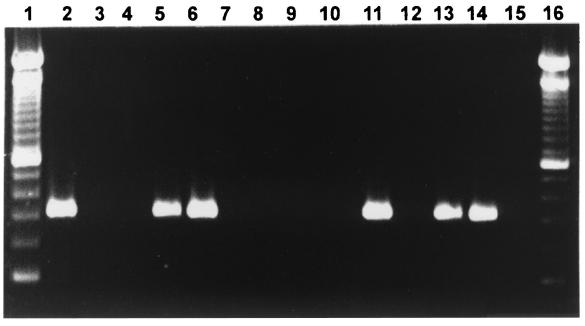

FIG. 1. PCR detection of *C. difficile* toxin B gene in clinical specimens using primers CDTB1 and CDTB2 on ethidium bromide-stained 1.4% agarose gel. Lanes 1 and 16, 100-bp DNA ladder; lane 2, positive control *C. difficile* ATCC 9689; lane 3, negative control *C. sordellii* V0606; lanes 4, 8, 12, and 15, sterile water; lanes 5, 6, 11, 13, and 14, positive specimens; lanes 7, 9, and 10, negative specimens.

clinical isolate of *Clostridium sordellii* V0606 and sterile distilled water) controls were added to each run. For the detection of inhibitors, 4 μl of each eluate extracted from all PCR-negative samples was spiked with 4 μl of eluate from a PCR-positive stool.

Analytical sensitivity. Aliquots of 0.1 ml of serial dilutions from 10^9 to 10^2 bacteria/ml, obtained from an overnight growth of *C. difficile* toxigenic strain ATCC 9689, were transferred into 0.9 ml of *C. difficile*-negative liquid stool. Concentrations of inoculated stools ranging from 10^8 to 10 bacteria/g of stool were obtained and tested with the cytotoxicity assay. Corresponding stool pellets obtained through the processing were tested with the PCR assay.

All specimens negative with the cytotoxicity assay (59 out of 118) were also negative with the PCR method. No amplification products were observed in PCR-negative samples (Fig. 1). Among the 59 cytotoxin-positive samples, 54 were PCR positive (Table 1) and generated a single and clear band at 322 bp. The PCR method had a specificity of 100% (95% confidence interval, 92.4 to 100) and a sensitivity of 91.5% (95% confidence interval, 80.6 to 96.8). A nested PCR, which is known to be a very sensitive technique (3, 4), was performed on the five PCR-negative cytotoxin-positive specimens. Three of them remained negative, suggesting that the cytopathic effect observed in the cytotoxicity assay was possibly due to the presence of cytotoxins other than *C. difficile* toxins. In the package insert of the assay used for the detection of cytotoxicity (*Clostridium difficile* Toxin/Antitoxin Kit), the possibility of a cross-reaction between *C. difficile* and *C. sordellii* is mentioned. Toxigenic isolates of *Clostridium sordellii* produce toxins HT and LT that are very similar to *C. difficile* toxins A and B, respectively (18). Furthermore, toxins HT and LT can be neutralized by antibodies directed against *C. difficile* toxins A and B (5, 19). For one of our three PCR-negative cytotoxin-positive specimens, which was also negative after a nested PCR, a positive culture for *C. difficile* was obtained. A PCR and a cytotoxicity assay were performed on this *C. difficile* isolate, and both results were negative. At least two strains of toxigenic *C. difficile* harboring variations of both toxin A and toxin B genes have been reported (21). A modification of the sequence of the toxin B gene (*tcdB*) could result in a negative PCR. A low number of *C. difficile* cells in the stool could be responsible for two of our PCR-negative cytotoxin-positive specimens since nested PCR was positive for both specimens. However, nested PCR is more time-consuming and more prone to contamination. Results from the different tests performed on the five discrepant specimens are summarized in Table 2. It is highly improbable that inhibitors were responsible for the five PCR-negative cytotoxin-positive specimens since none of these samples were found to contain inhibitors when spiked with *C. difficile* DNA. Although the commercial kit used in this study has been designed to purify high-quality bacterial DNA from stool specimens, our study is among the first to be published regarding its performance. Therefore, if this PCR assay is to be used as a routine diagnostic technique, each stool specimen should still be amplified in duplicate, spiking one of the duplicates with a standardized quantity of *C. difficile* DNA extracted from a toxigenic strain. The detection limit obtained with the PCR assay was 10^6 *C. difficile* cells/g of stools. This method was 10-fold more sensitive than the cytotoxicity assay which detected 10^7 *C. difficile* cells/g of stools. During the study, in addition to the stool pellets, 45 unprocessed portions of fresh stools were frozen upon arrival at the laboratory and also tested by PCR under the same conditions. No discrepancy was observed between results of the fresh stools and the pellets. In addition, the analytical sensitivities of the PCR assay performed on both stool pellets and fresh stools were identical.

We have developed a PCR assay for the detection of *C. difficile* in stool specimens that demonstrates an excellent specificity (100%) and a very good sensitivity (91.5%) when compared to the cytotoxicity assay. The PCR assay is much more

TABLE 1. Comparison of results of *C. difficile* PCR assay and cytotoxicity assay

PCR result	No. of samples that were cytotoxin:		Total
	Positive	Negative	
Positive	54	0	54
Negative	5	59	64
Total	59	59	118

TABLE 2. Analysis of the five PCR-negative cytotoxin-positive specimens

Specimen no.	Presence of *C. difficile* toxin				
	Cytotoxin assay[a]		Culture	Inhibitors	Nested PCR[b]
	24 h	48 h			
1	−	+	NA[c]	−	+
2	−	+	−	−	−
3	−	+	+	−	−
4	−	+	−	−	−
5	+	+	−	−	+

[a] Results were obtained after 24 and 48 h of cell culture inoculation.
[b] Based on work by Alonso et al. (3) with the following modifications: first amplification, primers CDTB1 and CDTB3 and 30 cycles of 94°C for 45 s, 54°C for 45 s, and 72°C for 75 s; second amplification: primers CDTB1 and CDTB2 and 30 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 30 s.
[c] NA, not available.

rapid since definite results can be obtained in 6 h, and it requires less technical manipulation than the cytotoxicity assay. Furthermore, according to the analytical sensitivity protocol used in this study, the PCR assay is 10-fold more sensitive. However, the PCR assay is more expensive, with reagent costs of approximately $6.00 per specimen compared to $1.80 for the cytotoxicity assay. In conclusion, the simple PCR assay developed in this study is very promising for the detection of *C. difficile* in stool specimens in the routine microbiology laboratory. This test would allow clinicians to obtain a result more rapidly, thus improving clinical management and judicious use of antibiotics.

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