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Rapid Detection of *Clostridium difficile* Toxin in Human Feces

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Fifty fecal specimens were tested by three methods, bacterial isolation, counterimmunoelectrophoresis, and tissue culture, for *Clostridium difficile* and its toxin. Ten specimens (20%) were positive by all three methods. An additional eight specimens were toxin positive only by counterimmunoelectrophoresis. Although counterimmunoelectrophoresis and tissue culture are of equivalent sensitivity, the additional dilution necessary for tissue culture assay may be critical when only small concentrations of toxin are present.

There have been recent reports on the etiology (1-3, 8, 11, 13, 15), diagnosis (5, 6, 19), and management (4, 9, 10, 14) of antibiotic-associated and non-antibiotic-associated (17) *Clostridium difficile* pseudomembranous enterocolitis (PME). Until now, *C. difficile* toxin could only be demonstrated by tissue culture response to fecal extracts or culture supernatant from *C. difficile* isolated from suspect feces. Welch et al. (18) have recently described a counterimmunoelectrophoresis (CIE) technique for detection of toxin in culture filtrates of *C. difficile*. They propose CIE as a means to identify the bacterium, not necessarily as a rapid diagnostic technique. Working simultaneously, we developed and now describe a rapid and sensitive immunochemical method, CIE, for detection of *C. difficile* toxin in human feces.

MATERIALS AND METHODS

Samples. Forty-eight fecal samples from 44 patients clinically suspected of PME were submitted to the clinical microbiology laboratory of the John Dempsey Hospital, University of Connecticut Health Center, for detection of *C. difficile* toxin. An additional two known toxin-positive fecal specimens were received through the generosity of Andrew Onderdonk, Boston, Mass. Specimens were transported to the laboratory at room temperature with no provision for oxygen exclusion. After inoculation of cycloserine-cefoxitin agar (19), used routinely for the isolation of *C. difficile*, the fecal sample, if solid, was diluted 1:2 in phosphate-buffered saline, centrifuged at 10,000 × *g* for 30 min, and filtered through a 0.45-μm-pore size membrane filter to remove cellular debris. Liquid feces were centrifuged and filtered without dilution. The filtrate was refrigerated at 4°C before analysis.

Tissue culture assay for *C. difficile* toxin. The assay protocol provided by the Anaerobe Laboratory, Virginia Polytechnic Institute, Blacksburg, Va., was modified as follows: 0.1 ml of serial 10-fold dilutions of filtrate (dilution range, 10^{-1} to 10^{-5}) was added to monolayer cultures of WI-38 cells purchased from Flow Laboratories, McLean,Va. A toxin standard purchased from the Anaerobe Laboratory, Virginia Polytechnic Institute, was used as a positive control and a fecal filtrate from an asymptomatic volunteer was used as a negative control. The tissue cultures were observed for the characteristic cytopathogenic effect (CPE) hourly for 8 h and again at 24, 48, and 72 h. Cytotoxicity was defined as the demonstration of CPE in at least 50% of the cells. All positive filtrates were retested and neutralized by the addition of 0.1 ml of a 1:25 dilution of *C. difficile* antitoxin purchased from the Anaerobe Laboratory, Virginia Polytechnic Institute. Neutralized cell cultures were examined at the same intervals as the non-neutralized cells.

CIE. Fecal filtrates were tested by CIE, using *C. difficile* antitoxin. A 10-μl sample of filtrate was added to the cathodic well of a 1% agarose-coated Mylar sheet (Bioware, Wichita, Kans.). Three millimeter-diameter wells were placed 10 mm apart. A 10-μl amount of antitoxin was placed in the anodic well. The same barbital buffer (pH 8.6; ionic strength, 0.05) was used both in the electrophoresis chamber and to prepare the agarose sheets. Sheets (5 by 7 mm) were electrophoresed in a cooled chamber (Bioware) for 45 to 60 min at 100 V and 35 to 40 mA. The power supply (Vokam) was run at constant voltage. After electrophoresis, the agarose sheets were inspected for a precipitin line between the antigen (toxin) and antitoxin wells. Both positive and negative controls were included with each procedure. All positive CIE tests were repeated, and the precipitin line was blocked by the addition of *C. difficile* antitoxin to the filtrate before electrophoresis.

All Mylar sheets were stained with Coomassie blue according to procedures outlined by Tilton (16).

Bacteriological identification. Suspicious colonies resembling *C. difficile* were subcultured from cefoxitin-cycloserine agar, repurified on 10% sheep blood agar, and identified by the API anaerobic biochemical strip (Analytab Products, Inc., Plainview, N.Y.). All isolates of *C. difficile* were tested for toxin production with the previously described toxin assay.

Inhibition of cytotoxicity by Lactinex. One gram of Lactinex (Hynson, Westcott and Dunning, Baltimore, Md.), a lyophilized suspension of *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*, was added to 100 ml of Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) and incubated under aerobic conditions for 72 h at 35°C. The suspension was centrifuged at 10,000 × *g* for 30 min, and the supernatant was filtered through a 0.45-μm-pore size membrane filter. A 0.9-ml portion of the sterile Lactinex filtrate and 0.1 ml of the serial dilutions (10^{-1} to 10^{-5}) of the control toxin were incubated at 37°C for 60 min. Toxin assays and CIE were performed. Controls included the addition of the sterile Lactinex filtrate directly to the tissue culture and the analysis of the Lactinex filtrate by CIE, using *C. difficile* antitoxin.

RESULTS

Table 1 presents the bacterial isolation, toxin assay, and CIE results on 18 positive and 32 negative specimens. Ten specimens from 10 patients were positive for the toxin by both CIE and tissue culture. Toxin was detected in five specimens from five patients by CIE but not by toxin assay. *C. difficile* was cultured from each of these 15 specimens. The sterile filtrates of these isolates subsequently produced characteristic CPE in tissue culture. In one patient, toxin was detected only by CIE. Two patients were initially positive by the three methods. Subsequent testing (5 days) of both patients revealed toxin only by CIE, but the organism was again isolated from both specimens and produced toxin after subculture.

Table 2 compares the sensitivity of CIE and the tissue culture toxin assay. At the recommended working dilutions of fecal filtrate (10^{-1} to 10^{-5}), there was no difference in sensitivity. Both methods failed to detect toxin at dilutions of 10^{-4} and 10^{-5}. Because of the additional dilution steps necessary when fecal filtrate is added to tissue culture, an initial dilution of 10^{-1} is equivalent to a final dilution in tissue culture of 5×10^{-3}. When fecal filtrates were tested undiluted by CIE and by tissue culture (final dilution, 5×10^{-2}), there was still no difference in sensitivity. However, in a number of patients, the undiluted fecal filtrate could not be tested because it was highly toxic to cells.

TABLE 1. *Comparison of bacterial isolation, CIE, and C. difficile toxin assay*

No. of specimens	Bacterial isolation[a]	CIE	Toxin assay
10	+	+	+
5	+	+	−
1	−	+	−
2 (repeat)[b]	(+)	(+)	(−)
32	−	−	−

[a] Culture positive = isolation of toxin-producing *C. difficile*.

[b] Two patients whose initial specimens were positive by the three methods and who on subsequent testing (+5 days) were positive by CIE and bacterial isolation.

TABLE 2. *Comparison of the sensitivity of CIE and tissue culture for the detection of C. difficile toxin*

Method	Result with dilution of *C. difficile* control toxin of:					
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Tissue culture	+	+	+	+	−	−
CIE	+	+	+	+	−	−

Sterile filtrates of Lactinex effectively blocked toxin-mediated CPE in WI-38 cells. When Lactinex was added to a 10^{-1} dilution of control toxin, approximately 10% of the cells showed rounding. Lactinex alone caused no characteristic CPE. Control toxin mixed with Lactinex was detected by CIE in dilutions of up to 10^{-3}. Lactinex alone showed no cross-reactivity.

All 18 patients whose feces contained the toxin of *C. difficile* showed evidence of severe enteritis, although sigmoidoscopy was not performed on all patients to confirm the diagnosis of PME. All positive patients were being treated with antibiotics at the onset of diarrhea. There was no additional clinical evidence of PME in the remaining 28 toxin-negative patients apart from diarrhea associated with antibiotic administration. The route of administration of the antibiotic, parenteral or oral, was not a factor in the development of *C. difficile* PME.

DISCUSSION

PME is a widespread disease associated with many factors, among them, antibiotic therapy. According to Fekety (7), antibiotic-associated PME can follow either oral or parenteral therapy, as well as short-term or prolonged antibiotic administration. Reports have confirmed PME after treatment with virtually any antibiotic, but most commonly ampicillin and clindamycin. A specific etiology, that is, toxin-producing *C. difficile*, has been shown by Bartlett et al. (1) to cause PME. Whether *C. difficile* PME is an acquired environmental infection or an ecological one in which the implicated organism overgrows the normal bowel flora and attaches to and multiplies on the colonic epithelia is still speculative. Patients with *C. difficile* PME respond to treatment with oral vancomycin, although there are instances in which relapses have been observed (4).

The laboratory diagnosis of PME is dependent

on the demonstration of the specific toxin by tissue culture or by isolation of *C. difficile* from the feces. Both techniques are time-consuming, and tissue culture requires specialized facilities, although the procedure is not technically difficult. There is a need for a rapid, specific, and sensitive assay for *C. difficile* toxin. The CIE method as described can be performed in any laboratory with little special equipment or training of technologists.

CIE is a rapid method (45 to 60 min) for toxin detection in fecal filtrates. Although a filtrate with a high toxin titer may cause CPE in tissue culture within 4 to 6 h, the tissue culture must be observed for 48 to 72 h before a negative report can be issued.

In all instances except one, those specimens in which toxin was detected by CIE also harbored *C. difficile* which produced characteristic toxin in subculture.

The one instance in which only the CIE was positive was a hospitalized patient clinically suspected of antibiotic-associated PME. After a time of protracted diarrhea, he was fed unpasteurized yogurt containing *L. bulgaricus* to reestablish normal gut microflora. His clinical condition improved. Isolation of *C. difficile* from his feces and demonstration of fecal toxin in tissue culture were unsuccessful. A recent report (L. Winans, Jr., G. B. Thornton, and T. R. Carski, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, A4, p. 1) indicated that the cytotoxic assay for *C. difficile* toxin could be blocked by Lactinex, a commercial preparation of *L. acidophilus* and *L. bulgaricus*. Preliminary experiments in our laboratory showed that the tissue culture toxin assay could be blocked by cell-free Lactinex preparations but CIE continued to detect the toxin. The mechanism of this toxin inhibition and the active component of Lactinex is not known. It has been reported by Taylor and Bartlett (12) that Cholestyramine and Colestipol, both anion-exchange resins, bind toxin or inhibit *C. difficile* toxic activity in tissue culture.

The experiment with cell-free Lactinex implies that, by a yet unknown mechanism, end products of *Lactobacillus* metabolism also prevent expression of *C. difficile* cytopathology in tissue culture but do not block the reaction of toxin with its specific antibody.

Under experimental conditions with the *C. difficile* toxin standard, the sensitivity of CIE is equal to that of tissue culture assay. However, in six patients, CIE proved to be more sensitive than tissue culture. When fecal filtrates are added to tissue culture, an "undiluted" specimen has actually been diluted 1:20 (1:2 in phosphate-buffered saline; 0.1 ml added to 0.9 ml of tissue culture). Fecal filtrates, however; can be added directly to the wells of the agarose sheet and electrophoresed. Specimens containing small concentrations of toxin may be diluted beyond the detection limits when being prepared for tissue culture assay, but produce precipitin lines with CIE because of the minimal dilution required (formed feces, 1:2; liquid feces, undiluted). There were two patients (Table 1) who initially showed toxin present by tissue culture, but repeat specimens taken subsequent to treatment showed small concentrations of toxin detectable only by CIE. The organism was isolated from the second set of specimens and produced toxin in subculture.

Thirty-two specimens from 28 patients were negative for *C. difficile* toxin by the three test methods. Although a large pool of "normal" feces has not been analyzed for CIE-reacting material, in only 1 of 18 specimens was CIE positive and both bacteriological and cell culture tests negative. Reasons have been postulated for the results in this patient. The precipitin reaction between control toxin, fecal toxin, and *C. difficile* antitoxin is also demonstrable in 24 h by passive immunodiffusion.

Antibiotic-associated PME is a life-threatening disease. Specific therapy, vancomycin, is available. The laboratory confirmation of active disease by recognition of *C. difficile* toxin or by isolation of toxin-producing *C. difficile*, both present in the feces, is necessary. Until more information is available on immunologically reactive, tissue culture-inactive *C. difficile* toxin, tests for toxin during the acute stages of disease should be confirmed by fecal isolation of toxigenic *C. difficile*.

It must also be recognized that detection of *C. difficile* toxin in or isolation of the organism from the feces may not constitute a specific diagnosis of PME. This anaerobic bacterium and its toxin could potentially be found in a wide variety of gastrointestinal diseases which are not histologically or endoscopically typical PME.

CIE offers potential advantages. It is economical, it is a widely used technique for the detection of other microbial antigens, and it can be completed in 60 to 75 min, including the filtrate preparation. CIE is sensitive, specific, and within the technical capabilities of most laboratories.

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