

## Identification of Toxigenic *Clostridium difficile* by Counterimmunoelectrophoresis

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A counterimmunoelectrophoresis (CIE) technique which reacted positively with culture filtrates of *Clostridium difficile* was developed and compared with a cytotoxicity assay in human embryonic lung cell cultures. CIE, employing *C. sordellii* antitoxin, detected 17 of 17 *C. difficile* strains. Of those positive by CIE, 13 were cytotoxic in cell culture. Fourteen *Clostridium* species other than *C. difficile*, *C. sordellii*, and *C. bifermentans* were negative by CIE. *C. sordellii* and *C. bifermentans* gave positive CIE results but were not cytotoxic. Similar sensitivity of toxin detection was observed for both methods. Optimal conditions for performing CIE included use of 48-h chopped meat-glucose broth cultures as the antigen source, use of a 10 $\times$ -concentrated U.S. Standard *C. sordellii* antitoxin, and electrophoresis for 1.5 h in 0.05 M tris(hydroxymethyl)aminomethane-barbital-sodium barbital, pH 8.8, at a constant current of 6 mA/slide. CIE appears to be a suitable alternative to the cytotoxicity assay and may serve as a means for presumptive identification of *C. difficile*.

*Clostridium difficile* has been documented as a cause of antibiotic-induced enterocolitis in experimental animals (2, 4, 5). Numerous observations in humans have also implicated *C. difficile* as the toxigenic agent primarily responsible for antimicrobial-associated pseudomembranous colitis (APMC) (3, 8, 10, 12). The laboratory diagnosis of APMC has been partially facilitated by the recent development of a selective and differential culture medium for isolation of *C. difficile* from fecal specimens (9). However, definitive diagnosis requires, in addition to clinical and bacteriological evidence, demonstration of the toxin and its neutralization by appropriate antitoxin in a cell culture assay. Chang et al. (6) have recently proposed a practical laboratory diagnostic method for APMC employing the cell culture assay. Due to the nonavailability in many laboratories of cell culture capabilities, we were led to investigate an approach to the laboratory diagnosis of APMC which would not require use of a cell line. We report here the development of a counterimmunoelectrophoresis (CIE) technique for identification of *C. difficile* and compare the results of the cell culture cytotoxicity assay with CIE for specificity and sensitivity.

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### MATERIALS AND METHODS

**Organisms.** Seventeen strains of *Clostridium difficile* isolated from humans were included in the study. Six strains were obtained from the Center for Disease Control, Atlanta, Ga., five were from the Wadsworth Veterans Administration Hospital, Los Angeles, Calif., three were from the Indiana University Medical Center, Indianapolis, Ind., and three were from the University of Utah Medical Center, Salt Lake City, Utah. Clinical manifestations associated with some of the isolates included pseudomembranous colitis (2), abscess (4), and other infections related to the gastrointestinal tract (6). Other *Clostridium* species were all recent clinical isolates obtained from the University of Utah Medical Center Anaerobe Laboratory. Identifications were performed by methods described in the Virginia Polytechnic Institute *Anaerobe Laboratory Manual* (11). Cultures were maintained at room temperature in tubes of chopped meat-glucose medium (Scott Laboratories, Fiskeville, R.I.).

**Cytotoxicity assay.** *C. difficile* toxin was demonstrated in monolayer cultures of WI-38 (human embryonic lung) cells maintained in Eagle minimal medium with 2% fetal bovine serum (Flow Laboratories, McLean, Va.). Toxin-containing filtrates were prepared from 24- to 72-h chopped meat-glucose broth cultures by sedimentation of the bacteria by centrifugation at 1,000  $\times g$  for 10 min and then passing the supernatant fluid through a 0.2- $\mu$ m membrane filter (Millipore Corp., Bedford, Mass.). A 0.1-ml amount of the broth or serially diluted filtrate in 0.85% saline was added to cell cultures which were incubated at 35°C in 5 to 10% CO<sub>2</sub>. The cells were examined microscopically for cytopathic effects at 4 h and again after 24 h of incubation. The endpoint for determining cytotox-

icity was the greatest dilution of the filtrate which affected at least 50% of the cells after 24 h of incubation. Neutralization of the cytotoxicity in cell cultures was performed by adding 0.1 ml of a 1:5 dilution of *Clostridium sordellii* antitoxin (U.S. Standard, Bureau of Biologics, Food and Drug Administration, Bethesda, Md.) to the tubes immediately before inoculation with toxin.

**CIE.** Toxin-containing culture filtrates, prepared as described above, were tested against *C. sordellii* antitoxin, which previously has been shown (5, 6, 14) to react with *C. difficile* toxin. The antigen (20  $\mu$ l) was applied to wells opposite the same volume of antitoxin on commercially prepared CIE slides (Meloy Laboratories, Inc., Springfield, Va.). U.S. Standard *C. sordellii* antitoxin was concentrated 10-fold by use of a minicon B-15 concentrator (Amicon Corp., Lexington, Mass.). Electrophoresis was performed at a constant current of 6 mA/slide in 0.05 M-tris(hydroxymethyl)aminomethane-barbital-sodium barbital buffer, pH 8.8, for 0.5 to 2.0 h. After electrophoresis, the slides were examined for precipitin lines with the aid of a viewer (Transidyne General Corp., Ann Arbor, Mich.).

## RESULTS

**CIE parameters.** CIE employing two different *C. sordellii* antitoxin lots, one containing 20 U/ml (U.S. Standard *C. sordellii* antitoxin lot no. S35), and the other containing approximately 5 U/ml (*C. sordellii* antitoxin lot 40067-3647), demonstrated negative results or barely visible precipitin lines with the *C. difficile* culture antigens. Samples of U.S. Standard *C. sordellii* antitoxin (20 U/ml) were concentrated 5-, 10-, and 20-fold and tested against *C. difficile* culture filtrates. Lines of precipitation significantly increased in density when the antitoxin was concentrated 10 or 20 times. Therefore, 10-fold-concentrated (200 U/ml) U.S. Standard *C. sordellii* antitoxin was subsequently employed as the antibody preparation for CIE. All of the *C. difficile* strains tested by CIE were positive when the antigen-containing filtrates were prepared from 48-h chopped meat-glucose broth cultures. Studies with strains that gave relatively strong reactions from 48-h cultures demonstrated that the minimum culture incubation time before CIE could be between 8 and 24 h. All strains tested after 4 and 8 h of incubation were negative, but subsequently gave positive or weakly positive results when tested by CIE at 24 h. To increase the likelihood of approaching maximal toxin titers, therefore, 48-h cultures were employed when preparing the antigen-containing filtrates for CIE. The optimal duration of CIE was determined by examining CIE slides at 0.5-h intervals during electrophoresis for the presence of precipitin lines. The antigen-antibody reaction with culture filtrates from some

representative *C. difficile* strains and concentrated *C. sordellii* antitoxin was first observed at 1.5 h. When the slides were examined at subsequent time intervals, no further enhancement of the reaction was detected. Therefore, electrophoresis was routinely performed for 1.5 h in all subsequent studies. Figure 1 illustrates representative CIE results showing the type of precipitin lines most frequently seen.

**Comparison of CIE and cytotoxicity assay.** The results of CIE and toxin testing in cell cultures of various *Clostridium* species are shown in Table 1. All 17 *C. difficile* strains were detected by CIE, but only 13 of 17 strains were toxin positive in cell cultures. Other *Clostridium* species positive in CIE but negative in the cell culture assay were one strain of *C. sordellii* and two strains of *C. bifermentans*. Twenty strains, representing 13 other *Clostridium* species, were negative in CIE. Three of the four *C. difficile*

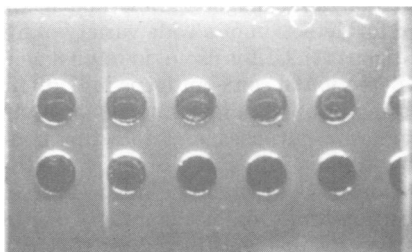


FIG. 1. CIE showing typical reactions of *C. sordellii* antitoxin with *C. difficile* culture filtrates. Electrophoresis, employing 20  $\mu$ l of the antibody and 20  $\mu$ l of the antigen preparation, was performed for 1.5 h in 0.05 M tris(hydroxymethyl)aminomethane-barbital-sodium barbital (pH 8.8) at a constant current of 6 mA per slide.

TABLE 1. Results of CIE and cell culture assay for detection of *C. difficile*

Organism	No. positive/no. tested <sup>a</sup>	
	CIE <sup>b</sup>	CC <sup>c</sup>
<i>C. difficile</i>	17/17	13/17
<i>C. sordellii</i>	1/1	0/1
<i>C. bifermentans</i>	2/2	0/2

<sup>a</sup> Culture filtrates of one strain each of *C. novyi* A, *C. novyi* B, *C. sporogenes*, *C. botulinum* A, *C. botulinum* B, *C. tetani*, *C. hastiforme*, *C. ramosum*, *C. glycolicum*; two strains of *C. tertium*; and three strains each of *C. paraputrificum*, *C. perfringens*, and *C. septicum* were negative in the CIE assay.

<sup>b</sup> CIE employing 20  $\mu$ l of broth culture filtrates as the antigen and concentrated *C. sordellii* antitoxin (20  $\mu$ l).

<sup>c</sup> Cytotoxicity in WI-38 cell cultures with 0.1 ml of broth culture filtrate. Positive results interpreted as tubes in which at least 50% of cells showed cytopathic effects.

strains that were negative by the cytotoxicity assay were weakly positive by CIE. These results suggested that CIE may be more sensitive than the cytotoxicity assay for detection of the toxin. Therefore, serial dilutions of the antigen (toxin)-containing filtrates were examined by both CIE and the cell culture assay. Table 2 shows results of CIE and the cytotoxicity assay examined at 4 and 24 h, employing 48-h culture filtrates of three strains. This method of comparison of CIE to the cytotoxicity assay for sensitivity indicated that CIE was equally or more sensitive than the cytotoxicity assay when the cell cultures were examined after 4 h of incubation, but the cytotoxicity assay was 2- to 10-fold more sensitive than CIE when the cell cultures were examined for toxicity after 24 h of incubation.

### DISCUSSION

CIE as a rapid diagnostic method has been applied for detection of a wide variety of microbial antigens (1). CIE is useful in rapid diagnosis, direct detection of some antigens in body fluids, monitoring of antigen levels semiquantitatively for prognostic purposes, and detecting non-viable or antibiotic-influenced organisms either in culture or in body fluids. The present study demonstrated four major advantages of CIE relating directly to identification of toxigenic *C. difficile* and the laboratory diagnosis of APMC. First, use of CIE may obviate the need to have access to mammalian cell cultures for toxin testing. This would be of particular value to laboratories not offering a virology service or not otherwise associated with a facility that routinely handles cell cultures. Second, some strains of *C. difficile* may be detected by CIE that

would not be positive in the cytotoxicity assay. It may be argued that strains demonstrating no toxicity in cell cultures are of little clinical significance. However, of the four strains that were positive in CIE but negative in cell cultures, all were felt to be potentially important clinically. Clinical conditions associated with these four isolates included clindamycin-associated diarrhea, necrotizing enterocolitis, rectal abscess, and an abdominal drainage. Third, identification of *C. difficile* may be accomplished by CIE rather than relying on biochemical testing. Although most strains are relatively homogeneous in biochemical characteristics and chromatographic products, usually an additional 48 h beyond primary isolation is required to confirm the identity of *C. difficile* isolates. Finally, CIE provides results within 2 h after obtaining the appropriate antigen preparation, whereas the cytotoxicity assay usually requires an additional 24 h before definitive results are obtained. The cytotoxicity assay is advantageous due to its demonstrated utility for toxin testing directly with stool specimen extracts. Further study needs to be undertaken to investigate the feasibility of CIE for direct evaluation of fecal specimens for *C. difficile* toxin.

The CIE precipitin reaction of *C. sordellii* antitoxin with *C. difficile* culture filtrates appears to represent a cross-reaction similar to the neutralization of toxin by this antitoxin observed in cell cultures (6). The positive CIE results with *C. sordellii* culture filtrates probably represent antibody specificity other than for cytotoxin since strains of this species were not toxic in cell cultures. Bartlett et al. (2) similarly demonstrated 15 strains of *C. sordellii* to be nontoxic in cell cultures.

We also observed positive CIE results with the two isolates of *C. bifermentans* tested. Although the explanation for this is not entirely clear, the antigenic similarity of *C. sordellii* and *C. bifermentans* has been documented. Ellner and Green (7) recognized four soluble antigens common to both *C. sordellii* and *C. bifermentans*. Also, both of our urease-negative strains originally identified as *C. bifermentans* should be classified as *C. sordellii* according to the criteria recommended by Nakamura et al. (13). Mannose was inhibitory or noncontributory to growth of these two strains, which Nakamura et al. consider to be characteristic of *C. sordellii* and not of *C. bifermentans*.

CIE with *C. sordellii* antitoxin may be questioned as a highly specific technique for identification of *C. difficile*. However, the use of the cycloserine- and cefoxitin-containing culture medium for isolation of *C. difficile* (9) can avoid this problem. It did not support the growth of

TABLE 2. Sensitivity of CIE compared to cell culture assay for detection of *C. difficile*<sup>a</sup>

Strain	CIE <sup>b</sup>	Cytotoxicity <sup>c</sup>	
		4 h	24 h
A	5	1	10
B	5	1	10
C	10	10	100

<sup>a</sup> Numbers represent reciprocal of greatest culture filtrate dilution giving positive results.

<sup>b</sup> CIE performed with a 48-h *C. difficile* broth culture filtrate (20  $\mu$ l) and concentrated *C. sordellii* antitoxin (20  $\mu$ l). Endpoints were determined as the greatest filtrate dilution which gave a visible line of precipitation.

<sup>c</sup> Cell culture assay performed by addition of 0.1 ml of a 48-h broth culture filtrate and examining cells for cytopathic effects after 4- and 24-h incubation. Endpoints were read as the greatest filtrate dilution affecting 50% of the cells.

the *C. sordellii* or *C. bifermentans* strains used in the present study. No colonies of these species were apparent on the medium after 24 h of incubation, whereas *C. difficile* colonies usually measured 2 to 3 mm in diameter when examined after 24 h of growth. In addition, specific antitoxin could most likely be prepared, thus avoiding the need to rely on a cross-reaction of *C. difficile* with *C. sordellii* antitoxin.

In conclusion, an alternative to the cytotoxicity assay for approach to the identification of toxigenic *C. difficile* may be proposed. After culture of specimens by the method of George et al. (9) for 24 h, colonies resembling *C. difficile* are transferred to chopped meat-glucose broth and incubated for 24 h. A portion of the growth is removed, and broth culture filtrates are prepared for use as the antigen source. Concentrated (10×) U.S. Standard *C. sordellii* antitoxin is employed as the antibody. Precipitin lines observed after 1.5 h of CIE confirm the isolation of *C. difficile*. Negative results after 24 h of incubation should be repeated at 48 and 72 h.

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