Problems Associated with Counterimmunoelectrophoresis Assays for Detecting *Clostridium difficile* Toxin

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The antitoxins currently used for the detection of *Clostridium difficile* by counterimmunoelectrophoresis react with other *C. difficile* antigens in addition to the toxins produced by the bacterium.

Clostridium difficile has been implicated as the primary cause of pseudomembranous colitis, which sometimes occurs in patients after treatment with antibiotics. The disease appears to be caused by the elaboration of toxins by *C. difficile* (2, 4, 7, 9, 10). Because of the severity of the disease, rapid methods for detecting *C. difficile* or its toxins are useful for a clinical laboratory. Currently, the presence of *C. difficile* in feces can be detected by isolation of the organism on a selective culture medium (8) or by neutralization of the cytotoxic activity with *C. difficile* or *Clostridium sordellii* antitoxin (5). Recently, counterimmunoelectrophoresis (CIE) has been proposed as a method for detecting the toxins (14, 15).

The first indication that a toxin was involved in pseudomembranous colitis was the neutralization of the cytotoxic activity of fecal filtrates by *C. sordellii* antitoxin from the U.S. Bureau of Biologics (USBB), Food and Drug Administration, Bethesda, Md. (4, 10, 13). Later, this neutralization was found to be a fortuitous cross-reaction in which USBB *C. sordellii* antitoxin neutralized the toxin produced by *C. difficile*. *C. difficile* antitoxin was not available at that time because *C. difficile* was not recognized as a pathogen. We have produced *C. difficile* antitoxin in both goats and rabbits and provide it to other laboratories (6).

Recent work by Bartlett et al. (3; N. S. Taylor, G. M. Thorne, and J. G. Bartlett, Clin. Res. **28:**285A, 1980) has demonstrated that *C. difficile* produces two toxins, and we have confirmed this. Toxin A has been described as an enterotoxin because it produces a positive response in ileal loops of rabbits (3). Both toxins cause rounding of several tissue culture cell lines (5), but toxin B has been designated the primary cytotoxin because of its much higher specific activity. Our laboratory has found that the cytotoxic activity of toxin B is approximately 1,000-fold greater than toxin A in culture supernatants of the 10 strains tested, in cecal contents of

hamsters with clindamycin-induced diarrhea, and in 6 stool samples from patients with antibiotic-associated diarrhea (N. Sullivan and J. Libby, unpublished results). Therefore, in cytotoxicity tests, toxin B masks the cytotoxic activity of toxin A.

The CIE tests that have recently been proposed as rapid methods for detecting *C. difficile* toxins (14, 15) have used either the USBB *C. sordellii* antitoxin or our *C. difficile* antitoxin. We suspect that the amount of each toxin present in most fecal specimens is not sufficient to be detected by CIE and that the precipitin bands that are obtained are due to other *C. difficile* antigens reacting with the antitoxin. Our findings in support of these beliefs are presented in this paper.

Our *C. difficile* antitoxin is prepared against a partially purified toxin preparation that contains several antigens of *C. difficile* (6). Several antibodies against these other antigens are present in the antitoxin as indicated by the multiple immunoprecipitation arcs obtained after crossed immunoelectrophoresis of crude toxin (dialysis culture supernatant) (1) with *C. difficile* antitoxin (6) (Fig. 1; see the figure legend for experimental details).

Because the *C. difficile* antitoxin contains antibodies against other *C. difficile* antigens, we obtained intense CIE reactions with strains of *C. difficile* that do not produce either toxin (Fig. 2). Both cytotoxicity and mouse lethality assays (1) were negative for these strains, and neither toxin was detected by polyacrylamide gel electrophoresis. These findings indicate that the CIE test can react with antigens that appear to be common to most, if not all, strains of *C. difficile*. We have also found that some strains of *C. sordellii* and *Clostridium bifermentans*, species which share cross-reacting antigens with *C. difficile* (12), produced precipitin bands when tested against the *C. difficile* antitoxin.

The potential of CIE for detecting cross-reacting antigens and, therefore, for yielding false
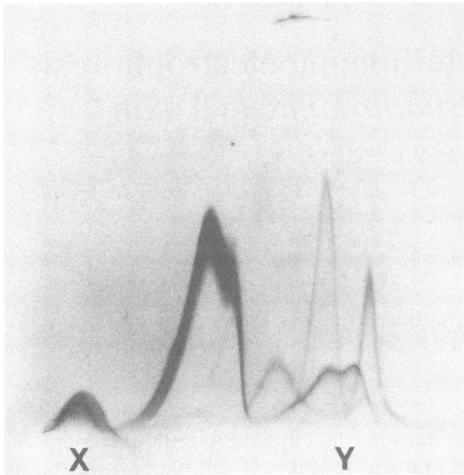
FIG. 1. Demonstration of multiple-precipitating antigen-antibody systems by crossed immunoelectrophoresis. For the first-dimension electrophoresis, 10 μl of C. difficile VPI 10463 dialysis culture supernatant (1) was placed in a well (4 mm in diameter) cut in a gel composed of 1.2% (wt/vol) low electroendoosmotic agarose (Sigma Chemical Co., St. Louis, Mo.) in 0.025 M Tris-Tricine buffer (pH 8.6) (10) and was subjected to electrophoresis (anode to right) at 9 to 10 V/cm (constant current) for 50 min at 10°C. For the second-dimension electrophoresis, the upper part of each gel (62.6 cm²) was composed of a 1.2% agarose gel (11 ml) containing 250 μl of goat antitoxin (6). Electrophoresis (anode at top of gel) was performed at 2 V/cm (constant current) for 18 h at 10°C. Toxin A forms the precipitin arc marked X, and toxin B forms one of the arcs at location Y (Lyerly et al., submitted for publication).

FIG. 2. CIE of toxin negative strains of C. difficile grown in brain heart infusion broth dialysis culture flasks (1) for 48 h. CIE plates (1% agarose in barbital buffer [pH 8.6], ionic strength, 0.05) were obtained from Meloy Laboratories Springfield, Va. Sample wells were 4 mm in diameter and 3 mm apart (edge to edge). Anodal wells (upper) contained 10 μl of goat antitoxin (6). Cathodal wells (lower) contained 10 μl of undiluted dialysis culture supernatant (1) from C. difficile strains VPI 2068, VPI 2618, VPI 2634, VPI 11183, and VPI 11186. Samples were subjected to electrophoresis in barbital buffer (pH 8.6) ionic strength, 0.05, at 10 V/cm (constant current) for 1 h at 10°C.

positive results has been confirmed in a recent clinical study. Crawford et al. (S. E. Crawford, P. Yungbluth, M. Sand, H. M. Sommers, Abstr. 3rd. Int. Symp. Rapid Methods Automation Microbiol, abstr. 109, 1981) found in a study of 78 patients with antibiotic-associated diarrhea that 50% of the positive samples detected by CIE were false.

When CIE was performed with either the goat or rabbit antitoxins (6) and crude C. difficile toxin (supernatant from C. difficile VPI 10463 dialysis culture [1], cytotoxicity titer of 10⁷), several precipitin bands formed. With the goat antitoxin (Fig. 3), strong precipitin lines appeared near the anodal well containing antibody. These precipitin lines decreased in intensity as the crude toxin was diluted and were only very faint at a 1:256 dilution. The rabbit antitoxin produced a similar pattern. With both antitoxins, a band was also present above the cathodal well containing antigen. We believe that this band is formed by toxin A. It is the only band present when purified toxin A (N. M. Sullivan,
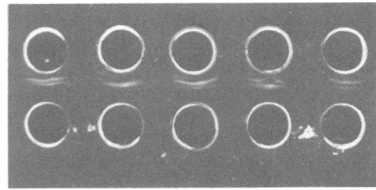
S. Pellett, and T. D. Wilkins, Infect. Immun., in press) is tested. It is also the only band present when crude toxin is tested against antitoxin which has been adsorbed with whole cells of C. difficile VPI 10463. This band is not present when partially purified toxin B (cytotoxicity titer of 10⁷) is tested, when crude toxin is tested against antitoxin which has been adsorbed with purified toxin A (Fig. 4), or in crude toxin preparations with cytotoxicity titers of less than 10⁷. When purified toxin A was tested against the goat antitoxin, the minimal amount of toxin detected was 30 μg/ml. This amount of toxin would result in a cytotoxicity titer of 10⁴; however, because of the 1,000-fold greater cytotoxicity of toxin B, a crude toxin preparation containing toxins A and B would have a cytotoxicity titer of 10⁷. Therefore, for toxin A to be detected by CIE, stool specimens from patients with antibiotic-associated diarrhea must have cytotoxicity titers of 10⁷ or greater. Of 18 toxin
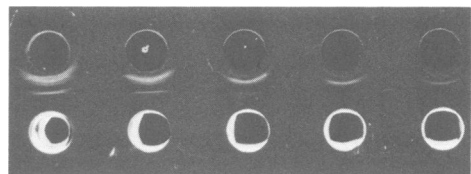

FIG. 3. CIE of C. difficile VPI 10463 crude toxin against C. difficile antitoxin (6). Anodal wells (upper) contained 10 μl of goat antitoxin (6). Cathodal wells (lower) contained, from left to right, 10 μl of undiluted dialysis culture supernatant (1) and twofold dilutions thereof in 0.05 M Tris-hydrochloride (pH 7.5). Electrophoresis conditions were as described in the legend to Fig. 2.
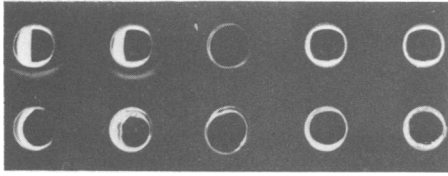
FIG. 4. CIE of *C. difficile* VPI 10463 crude toxin and toxin A against antitoxin adsorbed with toxin A. Anodal wells (upper) contained 10 μl of adsorbed goat antitoxin which was prepared by mixing equal volumes of antitoxin and purified toxin A (Sullivan et al., submitted for publication) and by incubating the toxin A-antitoxin mixture for 1 h at 27°C, followed by centrifugation at 12,800 × *g* for 10 min to remove antigen-antibody complexes. The supernatant was concentrated to the original volume by ultrafiltration (Minicon-A25, Amicon Corp., Lexington, Mass.). Cathodal wells (lower) contained, from left to right, 10-μl samples of crude toxin, a 1:2 dilution of crude toxin in barbital buffer, barbital buffer, toxin A, and a 1:2 dilution of toxin A in barbital buffer. Electrophoresis conditions were as described in the legend to Fig. 2.

positive stool specimens examined in our laboratory, most of the specimens had cytotoxicity titers of 10^3 and 10^4, and only 3 specimens had a titer of 10^6; therefore, these observations indicate that toxin A could not be detected by CIE in human fecal specimens with these techniques.

Toxin B has not been purified (Sullivan et al., in press). Therefore, we have not been able to determine if purified toxin B will react with the antitoxin in the CIE test. We have tested partially purified toxin B (cytotoxicity titer of 10^8) against goat immunoglobulin G specific for toxin B (D. M. Lyerly, S. E. H. West, and T. D. Wilkins, submitted for publication), and there was no reaction. We suspect that the amount of toxin B tested was too small to be detected by CIE.

CIE is clinically useful as a presumptive test for the rapid diagnosis of *C. difficile*-induced colitis. However, because the antitoxins currently used detect *C. difficile* antigens other than the toxins, positive results do not necessarily confirm the presence of *C. difficile* toxins in the sample. Therefore, we recommend that positive reactions be confirmed with the cytotoxicity test (5) which uses antitoxin that specifically neutralizes the *C. difficile* toxins. With the cytotoxicity test, false positive reactions occur very rarely, but we have found one strain of *Clostridium perfringens* that produces a cytotoxin which our antitoxin neutralized (N. Sullivan, unpublished results). Currently, we believe, in agreement with Ryan et al. (14), that the best method for

assessing the role of *C. difficile* toxins in pseudomembranous colitis is a combination of cytotoxicity testing (5) and direct isolation of the organism on a selective medium (8).

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