Problems Associated with Counterimmunoelectrophoresis Assays for Detecting Clostridium difficile Toxin

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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 celac 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diarrhea, and in 6 stool samples from patients with antibiotic-associated diarrhea (N. Sullivan and J. Libby, unpublished results).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...
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 10²), 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 10³; 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
positive stool specimens examined in our laboratory, most of the specimens had cytotoxicity titers of $10^5$ and $10^6$, 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 $10^6$) 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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**LITERATURE CITED**


