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
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 10^3), 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,
positive stool specimens examined in our laboratory, most of the specimens had cytotoxicity titers of $10^3$ and $10^6$, and only 3 specimens had a titer of $10^8$; 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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