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Evaluation of the Usefulness of Counterimmunoelectrophoresis for Diagnosis of *Clostridium difficile*-Associated Colitis in Clinical Specimens

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Results of counterimmunoelectrophoresis (CIE) were compared with those of isolation of *Clostridium difficile* and assay for cytotoxicity in HeLa cells. On the basis of 471 stool specimens, CIE exhibited a sensitivity of 38% and a specificity of 88% as compared with the cytotoxin assay. The predictive value of a reactive CIE results is low (17%), whereas the predictive value of a nonreactive CIE result is significant (96%) and therefore warrants its use as a screening test. In addition, stool filtrates may nonspecifically precipitate with the *C. difficile* antitoxin in the CIE test. Such nonspecific reactions may be identified by simultaneous electrophoresis against nonimmune serum.

Clostridium difficile and its toxin are central to the pathogenesis of antibiotic-associated diarrheal disease (AAD) (2, 3, 17). The laboratory diagnosis of AAD has been partially facilitated by the development of a selective and differential culture medium for isolating *C. difficile* from fecal specimens (8). In addition, demonstration of *C. difficile* toxin in fecal filtrates by a cell culture assay has been considered an important, although not definitive, diagnostic tool in the laboratory diagnosis of AAD (4, 7). Because of a lack of cell culture capabilities in many clinical laboratories, several investigators (10, 14, 18) have used a rapid and simple assay, counterimmunoelectrophoresis (CIE), for the detection of toxin in culture and fecal filtrates. A number of reports on the evaluation of the usefulness of CIE performed on clinical specimens have appeared, but the results from various investigations have not been in agreement.

The purpose of this study is to compare CIE with the cytotoxin assay and the bacteriological culture method. We report here the usefulness of CIE as a screening test for *C. difficile* toxin assay in a clinical laboratory.

MATERIALS AND METHODS

Stool specimens. A total of 471 stool specimens from 456 patients with a clinical diagnosis of antibiotic-associated diarrheal disease (AAD), based on history of antibiotic administration and altered bowel habits, were submitted to the Microbiology Laboratory at North Shore University Hospital, Manhasset, N.Y., for the isolation of *C. difficile* and the performance of CIE. Stool filtrates were prepared by the procedure outlined by Ryan et al. (14), and CIE was performed within 6 h of filtrate preparation. Stool filtrates, stored at −70°C for 1 month to 1 year, were thawed only at the time cytotoxin assay was performed.

CIE techniques. Both *C. difficile* culture filtrates, which were used as a positive control, and *C. difficile* antitoxin, which had been produced in goat serum, were purchased from the Anaerobic Laboratory, Virginia Polytechnic Institute, Blacksburg. According to the package insert, the standard *C. difficile* filtrate is cytotoxic at dilutions through 10^{-6} with Chinese hamster ovary cells. The goat antitoxin could be diluted to 10^{3} and would neutralize the cytotoxicity to 10^{-6} toxin. Twenty microliters of undiluted stool filtrate or positive culture filtrate control was applied to the cathodal well, and the same volume of antitoxin was applied to the anodal well of a 1% agarose-coated slide (10 by 7 cm). With each stool filtrate, nonimmune goat serum (Cappel Laboratories, Inc., Cochranville, Pa.) was used in parallel with goat antitoxin serum. Electrophoresis was performed at 30 mA for 30 min in barbital buffer (pH 8.2) having an ionic strength of 0.05. The slides were examined for precipitin lines immediately after electrophoresis and again after overnight incubation at 4°C. Nonspecific CIE reactions were defined as the forming of precipitin lines by stool filtrates with both nonimmune goat serum and antitoxin. Reactive denoted the presence of sharp, well-demarcated, single or multiple precipitin lines only in the presence of antitoxin. Those filtrates producing no bands or broad, diffuse precipitin bands were regarded as nonreactive.

Cytotoxin assay. The cytotoxicity of *C. difficile* toxin was assayed with monolayer cultures of HeLa cells (CCL-2; National Institutes of Health, Bethesda, Md.) maintained in minimal essential medium (Microbiological Associates, Walkersville, Md.) supplemented with 1% L-glutamine, 100 μg of streptomycin and 100 U of penicillin per ml, and 2% fetal calf serum in a 96-well

microtiter plate. Stool filtrates were initially diluted to 1:4 with phosphate-buffered saline (pH 7.2) and then were serially diluted either 2- or 10-fold with phosphate-buffered saline. A 0.1-ml amount of each dilution was added to each well. The microtiter plate was incubated at 35°C in 5 to 8% CO_2. The endpoint of cytotoxicity determination was defined as the highest dilution of the filtrate that caused 100% rounding of HeLa cells after 24 or 48 of incubation. Neutralization was demonstrated by preincubation of serially diluted stool filtrates with an equal volume of a 1:25 dilution of *Clostridium sordellii* antitoxin (Bureau of Biological Products, Bethesda, Md.) for 60 min at 35°C, followed by cytotoxicity assay. Reading of neutralization was made after 24 h of incubation. To store the results for an extended period, the microtiter plate was fixed and stained with crystal violet by the method of Gentry and Dalrymple (6).

Culture method. Stools were inoculated heavily onto cycloserine-cefoxitin-fructose agar (Remel, Lenexa, Kans.) and colistin-nalidixic acid agar (BBL Microbiology Systems, Cockeysville, Md.). Plates were incubated anaerobically at 35°C and examined after 48 to 72 h of incubation for characteristic *C. difficile* colonies (8). All presumptive *C. difficile* organisms were confirmed by API 20A (Analytab Products, Plainview, N.Y.) and gas-liquid chromatography (9).

RESULTS

Twenty-nine specimens (6.2%) were positive by the cytotoxin assay, Of these, 4 had toxin titers of between 10^2 and 10^3, 23 had titers of between 10^3 and 10^4, and 2 had titers of greater than 10^5.

The number of precipitin lines detected in reactive specimens with *C. difficile* antitoxin ranged from one to three. The culture filtrate used as a positive control showed two major lines, with a maximum total of five lines.

When stool filtrates were electrophoresed against nonimmune goat serum, our results revealed a high incidence (17.8%) of nonspecific reaction by CIE among clinical specimens (Table 1). All nonspecific specimens revealed single precipitin lines. In the majority of cases, the same intensity was observed with antitoxin as with nonimmune goat serum. The nonspecific precipitation reactions were also observed when the same stool filtrates were examined by CIE with nonimmune rabbit serum.

During the course of this study, it was noted that the intensity of precipitin lines of many reactive and nonspecific stool filtrates decreased with storage −70°C. Considering the impact of storage on the performance of CIE, we prospectively followed the changes of the precipitin lines with a few stool specimens for a 2-month period. Five stools, all CIE reactive, were chosen for this study. Two specimens, one positive for *C. difficile* isolation only and the other positive for *C. difficile* isolation and the cytotoxin assay, revealed an obvious loss of precipitation intensity over a 2-month period (Fig. 1). The remaining three reactive specimens maintained a similar degree of precipitation intensity throughout the study.

Table 2 shows the correlation between CIE and isolation in relation to the cytotoxin assay. CIE was found to have a very low sensitivity (38%) and an 88% specificity, based on the cytotoxin assay. Similar values were obtained when the results of *C. difficile* isolation were compared with those of the cytotoxin assay. Since cytotoxin was detected in only 18 (4%) of the 408 samples that were found to be nonspecific or nonreactive by CIE, we concluded that the most useful information obtained by CIE with fresh stool specimens is from a negative CIE result, based on a significant (96%) predictive value of a nonreactive CIE compared with its cytotoxin assay.

TABLE 1. Comparison of CIE, isolation, and cytotoxin assay in stool filtrates

CIE result	No. of specimens	No. positive in:		
		Isolation and cytotoxin	Isolation only	Cytotoxin only
Reactive	63	10	16	1
Nonspecific	84	0	3	2
Nonreactive	324	7	27	9

DISCUSSION

Several factors could contribute to the lack of a positive correlation between CIE and the

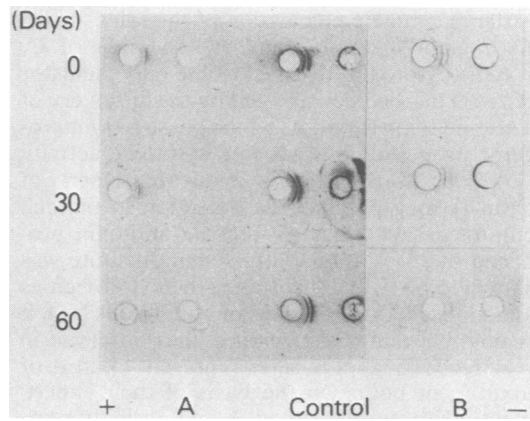

FIG. 1. Effect of freezing stool filtrates at −70°C on the reproducibility of CIE. Fresh stool filtrates were divided into three portions and stored at −70°C. CIE and cytotoxin assay were performed after 0, 30, and 60 days of storage at −70°C. CIE slides were dried and stained with Amido Black 10B. Specimen A was positive for *C. difficile* isolation but negative for cytotoxin assay. Specimen B was positive for both.

TABLE 2. Comparison of the significance of CIE and isolation in relation to cytotoxin assay[a]

Test	% Sensitivity	% Specificity	% Predictive value	
			Positive	Negative
CIE	38	88	17	96
Isolation	59	90	27	97

[a] For definitions, see reference 5.

cytotoxin assay. One possible factor is the lack of standard criteria for reading and interpreting CIE results. Since it was unknown which line(s) corresponded to *C. difficile* toxin(s), the presence of any line(s) was considered as a reactive result in our study. Reactive CIE results may be due to the presence of *C. difficile* toxin(s) or cell wall-associated antigens that are possibly common among related *Clostridium* spp. (13, 14). Another possible factor is the difference in the relative stability of *C. difficile* toxin(s) in stool filtrates as detected by immunological and biological assays. There was no loss of cytotoxin titer from stool filtrate after 4 months of storage at −70°C (data not shown). Storage at a freezing temperature (−70°C) appears to have a greater impact on the reproducibility of CIE than on that of the cytotoxin assay. It is unclear as to whether CIE results obtained from other investigations were from fresh or frozen stool filtrates. It is also conceivable that CIE results obtained with frozen stool filtrates would yield more true- and false-negative results in relation to the cytotoxin assays as compared with results obtained with fresh stool filtrates. A third possible factor is the incomplete elucidation of the roles of two *C. difficile* toxins, A and B. The role of *C. difficile* cytotoxin (toxin B) as the only mediator of AAD has been challenged by the discovery of a second toxin (toxin A) which possesses enterogenic properties but has low cytotoxic activity (1, 11, 12, 15, 16). The low cytotoxic property of toxin A may preclude its detection in the cell culture assay. Since *C. difficile* antitoxin produced by the Virginia Polytechnic Institute was shown to be able to neutralize the cytotoxicities and mouse lethality assays of both toxins (16), it is possible that the precipitin lines observed in reactive CIE stool filtrates represent toxin A or toxin B or both. On the basis of their experiments with purified toxin A and partially purified toxin B preparations, West and Wilkins (19) suggested that neither toxin A nor toxin B from clinical specimens can be detected by CIE. Their results obtained with partially purified toxin B preparations detected by cross-immunoelectrophoresis but not by CIE are contradictory, however, and remained to be clarified.

Our results indicate that stools analyzed by CIE may nonspecifically precipitate with *C. difficile* antitoxin. It is possible that a truly reactive type of precipitation is obscured by a nonspecific one. On the basis of the fact that there were only three specimens positive for *C. difficile* isolation (4%) and 2 positive by the cytotoxin assay (2%) in 84 nonspecific specimens, we conclude that stools with nonspecific CIE results can be considered to have a low likelihood of either the presence of *C. difficile* or a positive cytotoxin assay.

Ryan et al. (14) reported 100% sensitivity of CIE results in 50 specimens as compared with the cytotoxin assay. The difference observed between the sensitivity level of 38% obtained in our study and that obtained in their study may be attributed to a different selection of clinical specimens in our study. There was no information regarding sigmoidoscopy, colonscopy, or barium enema from our patient population. The routine staining used by Ryan et al. of the precipitin lines before examination may also account for the higher sensitivity of CIE results. Their level of specificity for CIE (80%) is close to that in our findings. Furthermore, Levine et al. (10) reported findings similar to ours (sensitivity, 25%; specificity, 76%) based on 37 clinical specimens.

George et al. (7) reported *C. difficile* recovery and toxin detection from 89 and 83% of stools, respectively, from patients with pseudomembranous colitis. The percentage of positive results was much lower in patients having AAD but lacking evidence of pseudomembranous colitis (*C. difficile* recovery, 58%; toxin detection, 26%). This finding suggested that neither the culture nor the cytotoxin assay could be considered as a highly specific and sensitive diagnostic test. These authors therefore suggested that the diagnosis of AAD should be based on the clinical setting, either a positive culture or a positive toxin assay and endoscopy or barium enema. On the basis of our findings, we suggest that CIE be used as a screening test in conjunction with culture in a clinical laboratory. A negative CIE result from a fresh specimen is a good indication of the absence of cytotoxic activity.

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LITERATURE CITED

1. **Bano, Y., T. Kobayshi, K. Watanabe, K. Ueno, and Y. Nozawa.** 1981. Two toxins (D-1 and D-2) of *Clostridium difficile* causing antibiotic-associated colitis: purification and some characterization. Biochem. Int. 2:629–635.
2. **Bartlett, J. G., T. Chang, M. Gurwith, S. L. Gorbach, and A. B. Onderlook.** 1978. Antibiotic-associated pseudomembranous colitis due to toxin-producing clostridia. N.

Engl. J. Med. **298**:531–534.
3. **Bartlett, J. G., N. Moon, T. Chang, N. Taylor, and A. B. Onderlook.** 1978. Role of *Clostridium difficile* in antibiotic-associated pseudomembranous colitis. Gastroenterology **75**:778–782.
4. **Chang, T.-W., M. Lauermann, and J. G. Bartlett.** 1979. Cytotoxicity assay in antibiotic-associated colitis. J. Infect. Dis. **140**:765–770.
5. **Galen, R. S., and S. R. Gambino.** 1975. Beyond normality: the predictive value and efficiency of medical diagnosis. John Wiley & Sons, Inc., New York.
6. **Gentry, M. K., and J. M. Dalrymple.** 1980. Quantitative microtiter cytotoxicity assay for *Shigella* toxin. J. Clin. Microbiol. **12**:361–366.
7. **George, W. L., R. D. Rolfe, and S. M. Finegold.** 1982. *Clostridium difficile* and its cytotoxin in feces of patients with antimicrobial agent-associated diarrhea and miscellaneous conditions. J. Clin. Microbiol. **15**:1049–1053.
8. **George, W. L., V. L. Sutter, D. Citron, and S. M. Fingold.** 1979. Selective and differential medium for isolation of *Clostridium difficile*. J. Clin. Microbiol. **9**:214–219.
9. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
10. **Levine, J. G., M. Kennedy, and J. T. LaMont.** 1982. Counterimmunoelectrophoresis vs. cytotoxicity assay for the detection of *Clostridium* toxin. J. Infect. Dis. **145**:398.
11. **Libby, J. M., and T. D. Wilkins.** 1982. Production of antitoxins to two toxins of *Clostridium difficile* and immunological comparison of the toxins by cross-neutralization studies. Infect. Immun. **35**:374–376.
12. **Lyerly, D. M., D. E. Lockwood, S. H. Richardson, and T. D. Wilkins.** 1982. Biological activities of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**:1147–1150.
13. **Poxton, I. R., and M. D. Byrne.** 1981. Immunological analysis of the EDTA-soluble antigens of *Clostridium difficile* and related species. J. Gen. Microbiol. **122**:41–46.
14. **Ryan, R. W., I. Kwasnik, and R. C. Tilton.** 1980. Rapid detection of *Clostridium difficile* toxin in human feces. J. Clin. Microbiol. **12**:776–779.
15. **Sullivan, N. M., S. Pellett, and T. D. Wilkins.** 1982. Purification and characterization of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**:1032–1040.
16. **Taylor, N. S., G. M. Thorne, and J. G. Bartlett.** 1981. Comparison of two toxins produced by *Clostridium difficile*. Infect. Immun. **34**:1036–1043.
17. **Viscidi, R., S. Willey, and J. G. Bartlett.** 1981. Isolation rates and toxigenic potential of *Clostridium difficile* isolates from various patient populations. Gastroenterology **81**:5–9.
18. **Welch, D. F., S. K. Menge, and J. M. Matsen.** 1980. Identification of toxigenic *Clostridium difficile* by counterimmunoelectrophoresis. J. Clin. Microbiol. **11**:470–473.
19. **West, S. E. H., and T. D. Wilkins.** 1982. Problems associated with counterimmunoelectrophoresis assays for detecting *Clostridium difficile* toxin. J. Clin. Microbiol. **15**:347–349.