JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1984, p. 923–926
0095-1137/84/110923-04$02.00/0

Vol. 20, No. 5

Criteria for Detection of *Clostridium difficile* Toxin Production by Counterimmunoelectrophoresis

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Received April 30 1984/Accepted 27 July 1984

Counterimmunoelectrophoresis (CIE) was compared with a cytotoxicity assay and isolation of toxin-producing *Clostridium difficile* for the diagnosis of antibiotic-associated diarrhea. Standardized criteria for CIE positivity were established as two major precipitin lines with specific *C. difficile* antitoxin. Using these criteria on 425 fecal specimens, we determined the sensitivity of CIE (80%) to be comparable to that of the cytotoxicity assay (82%). Both methods were highly specific. With carefully controlled techniques, CIE can be used in routine diagnostic laboratories to screen for toxigenic *C. difficile*.

The laboratory diagnosis of antibiotic-associated diarrhea rests primarily on the identification of *Clostridium difficile* and estimation of toxin production by this bacterium in feces or culture filtrates. The organism is isolated with reasonable ease on selective media, and there are a number of previously published techniques which have been reported to enhance recovery of *C. difficile* (2, 6, 9, 17). The investigation of *C. difficile* toxin production has been hampered in many laboratories because of a lack of facilities. The usual method has been to measure cytolytic activity by tissue culture assay (3–5), but many laboratories do not have this capability. More recently, counterimmunoelectrophoresis (CIE; 8, 11, 13, 15) and enzyme immunoassays (19) have been applied to detect *C. difficile* toxin in fecal and culture filtrates. Results of CIE studies have been conflicting, perhaps because of different methodologies and different interpretations of what constitutes a positive reaction (10, 12, 14, 16, 18). For example, Wu and Fung (18) interpreted any precipitin line as a positive reaction in their investigation.

The purpose of the present study was to establish criteria for reactivity of *C. difficile* toxin-antitoxin mixtures in agarose gels and to use such criteria to compare CIE with a tissue culture assay and isolation of *C. difficile*.

(Part of this work was presented at the 23rd Interscience Conference on Antimicrobial Agents and Chemotherapy, Las Vegas, Nev., 24 to 26 October 1983 [R. P. Rennie, J. Elliott, M. Nardini, and J. H. Thornley, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 1983, 776, p. 227].)

MATERIALS AND METHODS

Culture of fecal specimens. A total of 425 fecal specimens submitted from patients with diarrhea who were receiving antibiotics were examined for *C. difficile* and its toxins. Feces were plated directly on receipt in the laboratory onto *C. difficile* agar (Oxoid Canada, Nepean, Ontario) which contained 500 mg of cycloserine (Parke, Davis & Co., Detroit, Mich.) per liter and 16 mg of cefoxitin (Merck Frosst) per liter and onto Columbia agar base (Oxoid) with 5% horse blood. Cultures were incubated anaerobically for 48 h and thereafter were examined for colonies with irregular edges that fluoresced under long-wave UV light. Such colonies were confirmed as *C. difficile* by Gram stain and biochemical and gas chromatographic profiles (7). Other anaerobic gram-positive bacilli were also identified by the same techniques. Feces or fecal filtrates prepared first by dilution with 0.01 M phosphate-buffered saline (pH 7.3) were then stored at −70°C. These were tested within 1 week of preparation for toxin activity.

Cytotoxin assay. Neutralization assays for *C. difficile* cytotoxin were performed by methods outlined by Ehrich and co-workers (5). Toxin activity was measured in microtiter monolayer cultures of Chinese hamster ovary (CHO) cells maintained in Hams F-12 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 1% glutamine, 1% fetal bovine serum, 100 mg of streptomycin per liter, and 10^5 U of penicillin per liter. Fecal filtrates were serially diluted 10-fold with 0.01 M phosphate-buffered saline. Each dilution (20 μl) was added to wells that contained 180 μl of the maintenance medium, and 100% cytotoxicity (rounding of the cells) was estimated microscopically after 24 h of incubation at 36°C in 5% CO_2. A positive cytotoxicity test was considered at dilutions of 1:100 or greater. Neutralization of cytotoxicity was determined by mixing fecal filtrates with an equal volume of a specific *C. difficile* antitoxin (provided by T. Wilkins, Virginia Polytechnic Institute, Blacksburg) diluted 1:25 in phosphate-buffered saline. After 1 h of incubation of these mixtures at room temperature, 20 μl of each mixture was added to wells of the microtiter cell culture. The CHO cell cultures were incubated for 24 h at 36°C in 5% CO_2, and 90% neutralization of cytotoxic activity was estimated. *C. difficile* toxic culture filtrates from strain VPI 10463 with and without the addition of antitoxin were included as controls in each assay.

CIE. Fecal filtrates or filtrates of *C. difficile* cultures grown in cooked meat-glucose broth (Oxoid) for 48 h at 36°C were examined by CIE. CIE was performed on sheets (50 by 75 mm) of Gelbond agarose support film (Bioproducts, Rockland, Maine) coated with 1% high electroendosomosis agarose B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) prepared in barbital buffer (pH 8.6) (ionic strength, 0.05). Wells (diameter, 3 mm) were placed 2 mm apart (1). Next, 10 μl of *C. difficile* antitoxin diluted 1:8 in phosphate-buffered saline was added to each anode well, and 10 μl of each fecal or culture filtrate was then added to the cathode wells. The dilution of antitoxin was determined by CIE of a 10^{-4} dilution of *C. difficile* toxin against various dilutions of antitoxin. The 1:8 dilution was the highest dilution that produced clearly defined precipitin lines. Positive and negative control samples were included on each agarose plate. Electrophoresis was performed in the same barbital buffer for 1.5 h at a constant voltage of 100 V. After electrophore-

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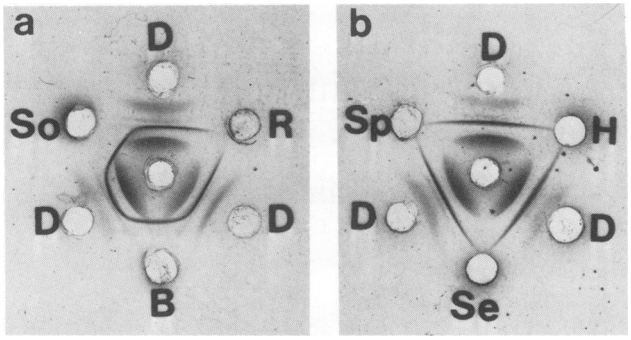

FIG. 1. Immunodiffusion of cooked meat culture filtrates of *Clostridium* species against *C. difficile* antitoxin. *C. difficile* (D), *C. sordellii* (So), *C. bifermentans* (B), and *C. ramosum* (R) are shown in a. *C. sporogenes* (sp), *C. septicum* (Se), and *C. histolyticum* (H) are shown with *C. difficile* (D) in b. *C. difficile* antitoxin is in the middle wells. The second major precipitin line with *C. difficile* filtrates is closer to the antitoxin well.

sis, the agarose sheets were inspected for precipitin lines between the cathode and anode wells. All agarose sheets were then washed overnight in successive changes of 0.03 M NaCl, 0.15 M NaCl, and distilled water. The sheets were then dried and stained with Coomassie blue.

Immunodiffusion studies. To elucidate the nature of the immunological reaction involved between toxigenic *C. difficile* culture filtrates and the *C. difficile* antitoxin, Ouchterlony-type immunodiffusion was performed with the same agarose and buffer system used for CIE studies. Immunodiffusion slides were held in a moist chamber for 24 to 48 h at room temperature. They were then inspected for precipitin lines, washed, and stained according to the procedure used for the CIE plates. Culture filtrates of at least two strains each of other *Clostridium* species, including *C. sordellii*, *C. histolyticum*, *C. septicum*, *C. ramosum*, *C. bifermentans*, *C. perfringens*, and *C. sporogenes*, were tested in these studies to determine the specificity of the *C. difficile* toxin-antitoxin reaction.

RESULTS

Immunodiffusion studies. Immunodiffusion was performed in the same agarose and buffer system as CIE to minimize differences between the two methods. Lines of identity were observed between the *C. difficile* antitoxin and culture filtrates of *C. sordellii* and *C. bifermentans* as well as *C. difficile* (Fig. 1). Culture filtrates of other *Clostridium* species did not show this reaction. However, both the *C. difficile* culture filtrates and fecal extracts that were cytotoxic in the CHO cell assay produced a second major precipitin line closer to the antitoxin well. Neither the second precipitin line nor cytotoxic activity was detected in culture filtrates of other species of clostridia tested.

Criteria for CIE positivity. The same collections of culture filtrates from several *Clostridium* species and cytotoxic fecal filtrates were also tested by CIE. Two major precipitin lines were apparent with *C. difficile* toxin-antitoxin mixtures, but only a single line was seen when the *C. sordellii* culture filtrate was electrophoresed with *C. difficile* antitoxin (Fig. 2). Cytotoxic fecal filtrates also showed two major precipitin lines. In a control experiment with *C. sordellii* antitoxin obtained from the Pasteur Institute, Paris, France, the *C. sordellii* culture filtrate produced only a single precipitin line, and a cytotoxic culture filtrate from *C. difficile* VPI 10463 did not show any precipitin lines by CIE or immunodiffusion with the *C. sordellii* antitoxin used either undiluted or at increasing dilution. This antitoxin was capable of neutralizing *C. difficile* cytotoxic activity in tissue culture but was 10- to 100-fold less potent than the *C. difficile* antitoxin. Three strains of *C. difficile* obtained from other institutions and a single strain from the present study that were all nontoxigenic by CHO cell assay produced only one precipitin band close to the anode well when tested with the *C. difficile* antitoxin. The criteria for a positive CIE reaction were therefore established as two major precipitin lines, using *C. difficile* antitoxin produced from strain VPI 10463 as the antibody.

Comparison of CIE, CHO cell assay, and isolation of *C. difficile*. With standardized criteria for a positive reaction by CIE, this method was compared with CHO cell assay and the isolation of either toxigenic or nontoxigenic *C. difficile* on 425 clinical specimens of feces. When compared with the isolation of *C. difficile*, CIE and CHO cell assay had equivalent sensitivities (80 and 82%, respectively), specificities (95 and 96%, respectively), and predictive values (63 and 62% positive and 98 and 99% negative, respectively). A total of 57 (13.4%) of the 425 fecal specimens were positive either by culture or by toxin assay. Of these, 31 (7.3%) were positive by both toxin assays and by culture, 18 (4.3%) were positive by both assays (but *C. difficile* was not grown), 7 (1.6%) were culture positive only, and only 1 was positive by CHO cell assay and negative by CIE. *C. difficile* was not isolated from this specimen, and the toxin titer was 1:100. Only one culture filtrate from the 38 *C. difficile* strains isolated was not cytotoxic by CHO cell assay.

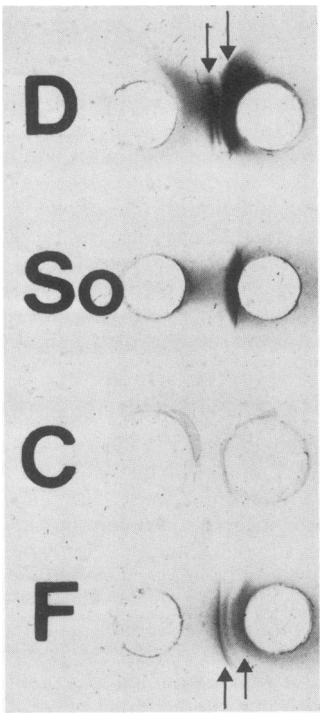

FIG. 2. CIE of *C. difficile* culture filtrate (D), *C. sordellii* culture filtrate (So), a noncytotoxic fecal filtrate (C), and a cytotoxic fecal filtrate (F). *C. difficile* antitoxin is in the anode wells. Arrows indicate the two major precipitin lines which denote a positive CIE reaction.

DISCUSSION

One of the major deterrents to CIE as a method of screening for cytotoxic *C. difficile* has been the lack of standardized criteria for interpreting positive reactions. Considering any precipitin line as a positive reaction, Wu and Fung (18) found 84 (18%) nonspecific reactions from 471 fecal specimens, but only 2 of these were cytotoxic by cell culture assay. Ryan and co-workers (11) did not note any nonspecific reactions among 48 fecal specimens examined by CIE and tissue culture assay, but they did not report on the criteria used for determining a positive reaction by CIE. Other investigators have attempted to draw conclusions on the value of CIE based on small sample numbers and without clearly defined criteria for positivity (8, 12).

The studies of West and Wilkins (16) suggest that the precipitin lines seen by CIE with *C. difficile* antitoxin may not be due to either of the toxins (A or B) known to be produced by the organism, whereas our results showed that only cytotoxic *C. difficile* culture filtrates and toxin-containing fecal filtrates produced the major precipitin lines seen by CIE. Noncytotoxic strains we have tested produce a CIE result similar to that observed with *C. sordellii*. Although we can make no final conclusions on the identity of the precipitin lines seen by CIE, it seems highly likely that one of these lines is associated with toxigenicity in *C. difficile*.

Our immunodiffusion and CIE results with other *Clostridium* species confirm and extend the work of others (15) that only *C. sordellii* and *C. bifermentans* exhibit cross-reactions in agarose gels with *C. difficile* antitoxin. We did not encounter any fecal filtrates that gave positive CIE reactions by our criteria from specimens in which clostridia other than *C. difficile* were isolated. Only those fecal filtrates that produced cytolytic activity neutralized by *C. difficile* antitoxin were found to produce two major precipitin lines by CIE. It is of interest to note that one fecal filtrate that showed a single strong precipitin line by CIE was not cytotoxic by CHO cell assay, and *C. difficile* was not isolated. According to our criteria for CIE positivity, this result was considered as negative. Subsequently, a histological diagnosis of hydatid disease was made. Such an observation reinforces the importance of clearly defined criteria for the estimation of a positive reaction by CIE.

The results given above indicate that measurements of *C. difficile* products that are either cytolytic or CIE positive are better predictors of a negative result than of a positive result when compared with the standard of isolation of the infecting organism from the clinical specimen. We believe that isolation of *C. difficile* should be the standard for comparing methods of measuring toxigenicity. The infectious process requires the presence of the organism even if it is not isolated from all clinical specimens. Toxigenicity should therefore be measured against that standard. The positive predictive value could perhaps be improved by placing strict control on acceptance of fecal specimens for investigation. Patients receiving other agents known to produce diarrhea in addition to antibiotics or patients who have known underlying chronic bowel disorders and are taking antibiotics might be considered separately. The extent and location of disease may well affect isolation rates of *C. difficile* and the appearance of significant amounts of toxin in feces. Also, the adjunct of significant sigmoidscopy findings and biopsy-proven pseudomembranous colitis would significantly improve the positive predictive values for these assays. We have yet to evaluate fully these various categories of patients.

In summary, the diagnosis of antibiotic-associated diarrhea caused by toxigenic *C. difficile* can be made with relative ease in routine diagnostic laboratories by culture of feces on selective media and CIE of fecal and culture filtrates. Standardization of the methodology is important, and potent *C. difficile* antitoxin is essential for reproducible results.

ACKNOWLEDGMENTS

We acknowledge the assistance of Tracey D. Wilkins at the Virginia Polytechic Institute for providing *C. difficile* antitoxin and toxin.

This study was supported in part by a grant from Oxoid (Canada).

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