Comparison of culture, cytotoxin assay and two EIA tests with clinical diagnosis of *Clostridium difficile*-associated diarrhea

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OBJECTIVE: The most common etiology of infectious diarrhea in hospitalized patients is *Clostridium difficile*. No single laboratory test yields a definitive diagnosis. Four methods were evaluated for their sensitivity and specificity in patients who had clinically defined *C difficile*-associated diarrhea.

METHODS: Clinical criteria for *C difficile*-associated diarrhea were defined. All adult in-hospital patients whose stools were tested for *C difficile* were prospectively followed. Stools were examined with culture on a selective medium, a commercial cytotoxicity assay (CTA), and two commercially available enzyme immunoassays (EIAS) for toxin A (Meridian) and toxin AB (CBC).

RESULTS: During the study period 235 stool specimens from 185 patients were tested. Fifty-one patients were positive for *C difficile* or its markers. CTA was most sensitive (80%), whereas CBC-EIA was most specific (98%). Differences in the sensitivities of CTA and Meridian-EIA were minor (80% versus 73.3%) and they were equally specific (95.5%).

CONCLUSIONS: The sensitivity and specificity of EIA for toxin A is similar to other tests. However, due to rapidity and ease of performance, it may be a more practical test for the diagnosis of *C difficile*-associated diarrhea, especially if the cytotoxin assay is not available. *(Pour résumé, voir page 164)*

Key Words: Clostridium difficile, *Culture, Cytotoxin, Diagnosis, Enzyme immunoassay*

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Comparaison des cultures, des dosages de cytotoxine et de deux épreuves d'immunoenzymologie dans le diagnostic de la diarrhée associée à *Clostridium difficile*

OBJECTIF : L'étiologie la plus fréquente de la diarrhée infectieuse chez les patients hospitalisés est *Clostridium difficile*. Aucune épreuve de laboratoire ne donne à elle seule le diagnostic définitif. Quatre méthodes ont été évaluées quant à leur degré de sensibilité et de spécificité chez des patients qui avaient déjà été identifiés comme cliniquement atteints de diarrhée associée à *C. difficile*.

MÉTHODES : Des critères cliniques ont été définis pour ce qui est de la diarrhée associée à *C. difficile*. Tous les patients adultes hospitalisés dont les selles ont été testées en vue d'un dépistage de *C. difficile* ont été suivis de façon prospective. Les selles ont été examinées par culture sur un milieu sélectif à l'aide d'une méthode courante de mesure de la cytotoxicité (CTA) et de deux épreuves d'immunoenzymologie (EIA) couramment employées pour dépister la toxine A (Meridian) et la toxine AB (CBC).

RÉSULTATS : Durant l'étude, 235 spécimens de selles provenant de 185 patients ont été testés. Cinquante-et-un patients se sont révélés positifs à l'égard de *C. difficile* ou de ses marqueurs. La CTA s'est révélée la plus sensible (80 %), alors que la CBC-EIA s'est révélée la plus spécifique (98 %). Les différences sur le plan de la sensibilité de la CTA et de l'EIA Meridian ont été mineures (80 % contre 73,3 %) et elles se sont révélées d'une égale spécificité (95,5 %).

CONCLUSION : La sensibilité et la spécificité de l'EIA pour le dépistage de la toxine A sont semblables à celles des autres épreuves. Toutefois, à cause de sa rapidité et de sa facilité d'exécution, elle pourrait s'avérer plus pratique pour le diagnostic de la diarrhée associée à *Clostridium difficile*, particulièrement si l'épreuve d'immunoenzymologie n'est pas disponible.

CLOSTRIDIUM DIFFICILE IS AN OPPORTUNISTIC PATHOGEN that can cause intestinal infection in any setting and following any procedure that destabilizes the normal protective intestinal flora. Antibiotic therapy is, by far, the most common predisposing factor, implicated in more than 98% of cases. The organism produces two toxins, an enterotoxin (toxin A) and a cytotoxin (toxin B) (1,2). Toxin A is believed to play the primary role in the pathogenesis of the disease because of its enterotoxicity. Toxin B is cytotoxic and possibly produces tissue damage after the initial action of toxin A.

C difficile is a major cause of hospital acquired diarrhea. Toxigenic *C difficile*, its toxins or both have been detected in 12 to 19% of all fecal specimens submitted to microbiology laboratories (3,4). The spectrum of disease caused by *C difficile* extends from asymptomatic carriage through mild self-limited diarrhea to severe pseudomembranous colitis. In the 1970s, detection of toxin B by tissue culture was shown to have a good correlation with the endoscopic finding of pseudomembranous disease. However, pseudomembranous disease is now rarely seen because most *C difficile* infections are diagnosed and treated earlier and endoscopy is not warranted (5). Consequently, in the recent past there have been very few reports in which endoscopy findings were used for diagnosis. Many have used the clinical criteria as a gold standard to assess the performance of *C difficile* diagnostic methods. Presently, five different types of tests are available to detect *C difficile* or its toxins in feces. We tested four assays and compared the results with the clinical diagnosis of *C difficile*-associated diarrhea.

MATERIALS AND METHODS

Stool specimens submitted to the Department of Microbiology, Victoria Hospital, London, Ontario from July 1, 1992 to December 31, 1992 were studied. Only adult patients with a hospital stay of more than 72 h were included. Stools were investigated for *Salmonella* species, *Shigella* species, *Yersinia* species and *Campylobacter* species, as well as for *Escherichia coli* O157:H7. In addition, stools were tested for the presence of *C difficile* and its toxins A and B. At the time of culture, a portion of each specimen was aliquoted and stored at –70°C for later cytotoxicity testing by tissue culture method.

***C difficile* culture:** Stools were subjected to alcohol shock treatment (6) to kill vegetative cells. After removal of alcohol by centrifugation, the pellet was used to inoculate a selective medium for *C difficile* containing D-cycloserine, cefoxitin and fructose (CCF medium; Oxoid, Unipath). The plates were incubated for up to 72 h at 35°C under anaerobic conditions. Spreading yellow colonies with ground glass appearance resembling *C difficile* were subcultured onto 5% horse blood plates containing Columbia base agar. One plate was incubated aerobically and the other anaerobically for 48 h. The anaerobic isolates were considered to be *C difficile* if they had typical colony morphology on the selective plates and produced butyric, isobutyric, valeric and isocaproic acid (7).

Toxin A by EIA (Meridian-EIA): Toxin A was detected by enzyme immunoassay (EIA) using a Premier kit (Meridian Diagnostics, Inc, Ohio). Stool specimens were prepared, processed and results were interpreted according to the instructions of the manufacturer. The kit employs polyclonal antitoxin A capture antibody adsorbed to breakaway microwells and requires 2.5 h to perform. Results were read visually.

Toxin AB by EIA (CBC-EIA): Cytoclone A+B (Cambridge Biotech Corporation [CBC], Massachusetts) was employed to detect toxins A plus B. Cytoclone is also an EIA and uses breakaway microwells coated with toxin specific monoclonal antibodies. Toxin-antibody complex is detected after washing, by conjugation with biotin-

TABLE 1
Statistical parameters of *Clostridium difficile* tests on 30/185 patients with *C difficile*-associated diarrhea

Test	Number positive		Sensitivity	Specificity	Predictive value	
	Total	True			Positive	Negative
Culture	41	23	76.7	94.8	56.0	95.4
Meridian-EIA	29	22	73.3	95.5	75.9	94.9
CTA	31	24	80.0	95.5	77.4	96.1
CBC-EIA	22	19	63.6	98.0	82.6	93.2

CBC Cambridge Biotech Corp; CTA Commercial cytotoxicity assay; EIA Enzyme immunoassay

tagged goat polyclonal antibodies specific for both toxins. The test was performed according to the instructions of the manufacturer and also took approximately 2.5 h to perform. Again results were determined visually.

Cytotoxicity assay (CTA): Cytotoxicity was tested using commercial tissue culture, Bartel Cytotoxicity Assay for *C difficile* (Baxter Health Care Corporation, Bartel Division, Washington). Tissue culture microtrays were stored at 35°C until used. Stool specimens frozen at –70°C were thawed and thoroughly mixed. A stool dilution was prepared and filtered through a sterile 0.45 µm filter. The filtrate was mixed with an equal volume of antitoxin. Tissue culture microwells were inoculated with 0.05 mL of filtrate or filtrate/antitoxin, making the final inoculum dilution 1:40. With every run, appropriate controls were used. The tissue culture tray was incubated at 37°C and inspected for specific, cytopathic effect (CPE) under a bright field microscope at 100x magnification at intervals from 4 to 6 h for up to 48 h. The result was considered positive if the patient's test well produced characteristic CPE but none was evident in the well with the stool filtrate and antitoxin. If both the patient's test well and control well showed CPE, a titration of stool filtrate was performed.

Chart review: The charts of all patients whose stools were tested were reviewed. The criteria used to determine if a patient had *C difficile*-associated disease were as follows: antimicrobial therapy in the past two months; clinically significant diarrhea, ie, three or more loose stools in a 24 h period; clinical response to oral vancomycin or metronidazole therapy; absence of inflammatory bowel disease and failure to isolate alternate enteric pathogens; presence of *C difficile*, or one or more of its markers; and sigmoidoscopic finding compatible with pseudomembranous colitis. Patients with the first four criteria and with *C difficile* markers or sigmoidoscopic finding of pseudomembranous colitis were considered to have *C difficile* disease.

RESULTS

Patients included in this study were all 18 years of age or older. During the study period of July 1 to December 31, 1992, 235 stools samples from 185 adult in-hospital patients were received. Fifty samples were repeat specimens from 17 patients who were negative for *C difficile* markers. It is our policy not to test repeat specimens from patients who are positive for any of the *C difficile* markers. One hundred and two patients had significant diarrhea as defined by this study. One hundred and sixteen patients had received antibiotics previously. None of the stool samples were positive for *Salmonella* species, *Shigella* species, *Yersinia* species, *E coli* O157:H7 or *Campylobacter* species. Of 185 patients 51 were positive for *C difficile* or its markers. *C difficile* was isolated from the stools of 41 patients. Toxin B was detected in stool samples of 31 patients by CTA. The Meridian-EIA detected toxin A in 29 patients, whereas CBC-EIA was positive in only 22 patients. Eighteen patients were positive for all *C difficile* tests. The study included only two patients who underwent sigmoidoscopy. Neither had visual or histological evidence of pseudomembranous colitis. Using clinical and diagnostic criteria, 30 patients were considered to have diarrhea due to *C difficile*. Of 155 patients considered not to have *C difficile*-associated diarrhea, four met all four clinical criteria. Of 30 patients with *C difficile* disease, 23 were culture-positive, 24 were positive by CTA, 22 by Meridian-EIA and 19 were positive by CBC-EIA. Sixteen of the 30 patients were positive by all four tests. Since repeat specimens from patients with positive markers were not tested, multiple specimens from negative patients were disregarded and were not included in the calculations. Sensitivities, specificities, and positive and negative predictive values of each test are shown in Table 1.

In patients who had diarrhea but were negative for *C difficile* markers, diarrhea was attributed to hyperalimentation in 39 cases, ulcerative colitis or Crohn's disease in six, gastrointestinal malignancies in 10 and necrotizing colitis in one.

DISCUSSION

The investigation of fecal specimens from patients suspected of *C difficile*-associated diarrhea has become a major task for most clinical laboratories. A simple, rapid and reliable test for *C difficile* is highly desirable. Currently, five kinds of commercial tests are available as diagnostic aids for *C difficile* infection: culture, latex agglutination and toxin assay by tissue culture, EIA and dot immunobinding assay.

C difficile culture requires up to three days for results. Compared with clinical criteria, the culture tech-

nique has been shown to be the most sensitive (4,8-11) though this was not our experience. False positive rates with this test are high as *C difficile* can be recovered from a high proportion of hospitalized patients receiving antibiotics without evidence of disease (12,13). Two-thirds of all cases of antibiotic-associated diarrhea are not due to *C difficile* (14) and it appears that in the absence of detectable toxin, the organism does not cause disease (2,15). In addition, a significant proportion of *C difficile* isolates, up to 40%, from hospitalized patients are nontoxigenic (4,8,16). Culture, like the latex agglutination test, does not differentiate between toxigenic and nontoxigenic strains of C *difficile*.

Earlier studies using endoscopy demonstrated a good correlation between CTA and pseudomembranous colitis (13,17,18). However, the results of a cytotoxin assay depend on the severity of the disease. CTA is positive in over 90% of patients with pseudomembranous colitis (19,20) and the toxin detection rate in fecal filtrates increases with the severity of the disease (21). It is, however, the less severe disease that poses a diagnostic dilemma. The sensitivity of CTA has been reported to range between 67 and 78%, using clinical criteria similar to ours (4,8-11). Contrary to the findings of most investigators, in the present study CTA was more sensitive than culture. In this regard our findings are not unique. DiPersio and investigators (22) found that among patients with clinical *C difficile*-associated diarrhea, CTA detected more cases than any other test, including culture. Furthermore, our sensitivity of 80% was comparable to previously reported rates. Many factors including final dilution of inoculum, age and type of the cell line employed influence CTA results (23-25). As we batched our specimens and the cell cultures were fresh, it is doubtful that the results of CTA could be duplicated in the routine laboratory setting. Repeat incubation of microtrays causes progressive deterioration of cell lines. This may cause false negative results towards to the end of the shelf life, as the cells may not be viable.

Since 1988, enzyme immunoassays for *C difficile* toxin A have been commercially available. At present, only Cambridge Biotech Corporation markets an EIA test that detects both toxins, A and B. Practically all strains of *C difficile* tested to date produce either both toxins or no detectable toxin (2,26). Therefore, for diagnostic purposes it matters little which toxin one detects. CTA detects toxin B, which is 1000–fold more cytotoxic than toxin A. CTA is also more sensitive than EIA, as it can detect picogram amounts of toxin B, whereas EIA tests require 1 to 10 ng of toxins/mL for detection. EIA results are also dependent on the quality and specificity of antibodies used for ligand capture (2). Indeterminate tests, although not encountered in this study, can be a problem. Doern and coworkers (27) found the CBC-EIA to be more sensitive than the Meridian-EIA: 84.5% versus 69%. Their criteria of *C difficile*-associated diarrhea were different from ours and were based on the results of four assays, in addition to clinical assessment of patients. It should also be noted that in their study, nine specimens yielding false negative results on initial testing with Meridian-EIA tested positive with this assay on repeat testing. Sensitivity and specificity of the Meridian-EIA in the present study are similar to those found by DiPersio et al (22).

In conclusion, although not shown by our study, CTA is considered the single most useful laboratory test for the diagnosis of *C difficile*-associated diarrhea. In our hands the Meridian-EIA was only slightly less sensitive. Of 30 patients with *C difficile*-associated diarrhea, Meridian-EIA detected only two fewer patients than CTA and was equal to CTA in specificity. The CBC-EIA was the most specific test, but least sensitive. Though the data are based on a relatively small number of patients with *C difficile*-associated diarrhea, it appears that the Meridian-EIA can be used as a primary test for detecting *C difficile*-associated diarrhea, if CTA is not available. In addition, EIA has the advantage of results being available in a short time. Regardless of the method of diagnosis, stools from patients without diarrhea and no history of previous antibiotic therapy should not be tested for *C difficile*. Due to frequent colonization of hospitalized patients with this organism, results from patients without symptoms are likely to be less specific.

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