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Laboratory Diagnosis of *Clostridium difficile*-Associated Diarrhea and Colitis: Usefulness of Premier Cytoclone A+B Enzyme Immunoassay for Combined Detection of Stool Toxins and Toxigenic *C. difficile* Strains

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Detection of *Clostridium difficile* toxins A and B in stools by Premier Cytoclone A+B enzyme immunoassay (EIA) was compared with detection by stool culture for *C. difficile* followed by detection of toxigenic isolates using the same EIA. Chart reviews were performed to evaluate the likelihood of *C. difficile*-associated diarrhea and colitis (CADC) for all patients with at least one positive toxin assay. While the toxins were detected in 58 of 85 consecutive CADC patients by both assays, CADC in 5 patients was detected only by stool toxin assay, and in 22 patients CADC was detected only by toxigenic culture. Our results suggest that for laboratories using a rapid toxin A+B EIA, direct toxin detection in stools should be combined with toxigenic culture in cases in which there is a negative stool toxin assay.

Clostridium difficile causes mainly nosocomial enteric diseases that range from antibiotic-associated diarrhea to pseudomembranous colitis (PMC) (13). Various laboratory methods may be used to diagnose *C. difficile*-associated diarrhea and colitis (CADC), but the two main approaches currently available are based on detection of toxin A, toxin B, or both in stool specimens and detection of toxigenic strains after stool culture (toxigenic culture) (6, 7, 11, 19). Although different data support the idea that both methods are necessary for optimal diagnosis of CADC (4, 5, 10–12, 18, 19, 21), the value of toxigenic culture is still a topic of debate (6, 20). Toxin A-negative, toxin B-positive, and both toxin A-positive and toxin B-negative *C. difficile* strains may be pathogenic in humans (1, 8, 15). Thus, methods used for toxin detection in stools and/or detection of toxigenic strains should ideally detect both toxins (14). For toxigenic culture, such an approach has been realized until now in only a few studies using both a toxin A enzyme immunoassay (EIA) and a cytotoxin assay (4, 10, 21). The cytotoxin assay is considered the most sensitive method for detecting toxin B in stools, but it requires the use of cell culture and antitoxin and is not well standardized. This limits its use in many clinical laboratories. Commercially available rapid EIAs that detect both toxins have been shown to accurately detect toxins in stools (3, 9, 16, 17) and may represent a more practical method among diagnostic strategies that combine detection of toxins in stools with toxigenic culture.

The aim of this study was to evaluate the usefulness of such a test, the Premier Cytoclone A+B EIA (Meridian Diagnostics, Inc., Cincinnati, Ohio), in identifying CADC patients by stool toxin assay and/or toxigenic culture.

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A total of 1,104 consecutive diarrheal stool samples obtained from 720 patients in our hospital was sent to our laboratory with a request for *C. difficile* toxin detection and tested by all assays in parallel. Specimens were processed immediately or stored at 4°C for ≤48 h prior to assay. Part of each sample was set aside and kept at −80°C for later follow-up testing if required. Toxins were detected in stools by the Premier Cytoclone A+B EIA, which is a rapid EIA that utilizes microwells coated with toxin A- and B-specific monoclonal antibodies. The test was performed according to the manufacturer's instructions. Positive and negative controls were included with each assay. Absorbance was read spectrophotometrically at 450 nm within 15 min. The absorbance values (optical density [OD]) were interpreted according to the manufacturer's instructions as follows: OD $<$ 0.200, negative; 0.200 $\leq$ OD $<$ 0.250, indeterminate; OD $\geq$ 0.250, positive. A portion of each specimen was inoculated onto brain-heart infusion agar supplemented with 5% sheep blood, 0.1% sodium taurocholate, cycloserine (250 mg/liter), and cefoxitin (8 mg/liter) (4). Plates were incubated in an anaerobic chamber for 48 h at 35°C. Colonies that were suspected of being *C. difficile* on the basis of characteristic morphology, odor, and Gram stain morphology were identified using conventional biochemical methods (2). All isolates were negative for lipase, lecithinase, and indole production as well as for milk digestion. These isolates were positive for gelatin and esculin hydrolysis and fermented glucose and mannitol but did not ferment maltose or sucrose. The colonies were subcultured (three colonies/culture-positive sample) anaerobically onto Wilkins Chalgren agar supplemented with 5% sheep blood for 24 h at 35°C. Colonies obtained by subculture were mixed with 100 μl of kit diluent before being tested for toxin production in the same way as for stool samples. When a specimen yielded a positive result with at least one toxin assay, the patient's charts were reviewed, and the patient was evaluated for the likelihood of having CADC,

TABLE 1. Analysis of clinical data of patients with positive direct toxin assay in stools and/or toxigenic culture

Direct toxin assay result	Toxigenic culture result	No. of specimens (no. of patients) from patients with clinical probability of CADC			Total true cases detected (%)
		Unlikely	Probable	Definite	
Positive	Positive	0	74 (51)	8 (7)	68.2
Positive	Negative	0	7 (4)[a]	3 (1)[a]	5.9
Negative	Positive	4 (4)	31 (19)	3 (3)	25.9

[a] All specimens were culture negative.

using slightly modified criteria of Peterson et al. (18). Clinical likelihood of CADC was considered as probable if all of the following criteria were met: (i) presence of diarrhea, defined as at least three stools per day for at least 48 h, (ii) antimicrobial therapy within 8 weeks of the onset of symptoms, (iii) absence of another recognized cause of diarrhea, and (iv) improvement of symptoms in response to therapy with metronidazole or vancomycin or after antibiotic withdrawal. If PMC could also be demonstrated by colonoscopy, the clinical likelihood was considered definite.

Of the 1,104 specimens tested, results for 130 (89 of 720 patients [12.4%]) were positive by stool toxin assay and/or toxigenic culture. Of the remaining 974 specimens, stool culture yielded nontoxigenic isolates of *C. difficile* in 19 from 12 patients. Cultures containing both toxin-positive and toxin-negative strains were not observed. Both direct toxin detection and toxigenic culture produced positive results for 82 specimens (58 of 89 patients [65.2%]). There were 38 specimens (26 of 89 patients [29.2%]) with stool toxin-negative and toxigenic culture-positive results. Ten specimens (from 5 of 89 patients [5.6%]) were positive only when tested by stool toxin assay, whereas culture results were negative. EIA indeterminate results were found in 22 of 1,104 specimens (2%) and 2 of 139 (1.4%) *C. difficile* strains. By repeating the EIA, all of the indeterminate results were resolved: results were considered to be positive for three specimens and one strain and negative for the remaining specimens and strains. Charts could be reviewed for all patients with at least one positive toxin assay (Table 1). These patients consisted of 37 males and 52 females (mean age, 61 years; range, 2.5 to 99 years). The clinical likelihood of CADC was considered unlikely for four patients aged 6, 22, 78, and 87 years because of rapid and spontaneous improvement of symptoms. Among the other 85 patients, 18 underwent colonoscopy, which permitted the diagnosis of PMC to be established in 11 cases. Patients for whom colonoscopy did not demonstrate PMC were all considered as having probable CADC. One child <3 years of age (2.5 years) was considered to have probable CADC. With the "gold standard" of stool toxin assay and/or toxigenic culture producing positive results and chart review providing evidence of probable or definite CADC, the sensitivities and specificities for direct toxin assay, toxigenic culture, and direct toxin assay plus toxigenic culture were 74.1 and 100%, 94.1 and 99.3%, and 100 and 99.3%, respectively.

When chart reviews were performed to establish diagnoses of CADC, the previously reported sensitivities and specificities of the Cytoclone A+B EIA ranged from 75.5 to 84.5%, and from 97.8 to 100%, respectively (4, 9), whereas the previously reported sensitivities and specificities of toxigenic culture ranged from 94.7 to 96.4% and from 98.6 to 99.1%, respectively (4, 10). These performances are comparable to those observed in our study. Our evaluation also confirms earlier data indicating that stool culture followed by toxin determination on isolates will result in a significantly higher number of CADC cases being detected than when the same toxin assays are performed directly on stools (4, 5, 10, 11, 18, 19, 21). In our study, 63 of the 85 CADC patients (74.1%) could be identified using stool toxin assay, but 22 CADC patients (25.9%), including 3 patients with PMC, would have been missed if toxin detection in stools had been used alone. It is conceivable that testing multiple specimens on patients belonging to this group might improve the detection rate of the stool toxin assay. Only three of these patients had multiple specimens (three to five specimens per patient), obtained over a 4- to 8-day period. However, all of these specimens were negative when tested by the stool toxin assay. In our study, five CADC patients had toxin-positive but culture-negative stools (10 specimens). This result may be explained by sampling problems inherent to the uneven distribution of *C. difficile* in the fecal samples but also, for three of these patients (seven specimens), by the fact that they were already on therapy when the stools were collected (7).

The major objection made against the use of toxigenic culture as a diagnostic tool is that toxigenic culture-positive/and stool toxin assay-negative patients may be asymptomatic carriers (20). Unfortunately, it is difficult to have a clear idea on this point since in most of the studies in which toxigenic culture was shown to be more sensitive than stool toxin assay, clinical data were incomplete or partially unavailable. In the present study, the analysis of all of the charts of patients with at least one positive test showed that only four patients, who all tested negative with stool toxin assay and positive with toxigenic culture, were considered to be carriers. It has been suggested that the detection of toxins in stools by EIA, coupled with testing strains for toxigenicity only in those cases in which direct toxin assay produces negative results, may be a satisfactory strategy, especially in laboratories without tissue culture facilities (7). Such an approach would have allowed us to reduce the number of strains tested for toxigenicity from 139 to 57 without alteration of the CADC detection rate. Rapid diagnosis of CADC is required in order to initiate specific antibiotic treatment and to take adequate measures to control nosocomial spread. Direct toxin detection in stools is obviously a more rapid method than toxigenic culture, results of which may, however, be obtained more rapidly, within approximately 76 h, when colonies are tested using a rapid toxin EIA. Thus, Premier Cytoclone A+B represents a helpful and practical test, which can be used both on stools and secondarily on colonies for routine investigation of antibiotic-associated diarrhea.

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