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Development of a Rapid Enzyme Immunoassay for *Clostridium difficile* Toxin A and Its Use in the Diagnosis of *C. difficile*-Associated Disease

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A rapid (2.5 h) direct enzyme immunoassay (EIA) for *Clostridium difficile* toxin A was developed for clinical use. Specimen centrifugation and filtration were not required. The EIA detected toxin A levels in patient stool as low as 20 pg (2 ng/ml of stool). The test was 5,000 times more sensitive for toxin A than it was for toxin B and did not react with a panel of other bacterial species with the exception of one highly toxigenic strain of *Clostridium sordellii*. The EIA was compared with the cytotoxin assay, culture of toxigenic *C. difficile* (toxigenic culture), and latex agglutination by using 313 fresh stool specimens submitted from patients with suspected *C. difficile*-associated disease. Results read visually and with a plate reader were similar. Sixty-two specimens were positive by one or more tests, but only 22 (35%) were positive by all four laboratory methods. The EIA was 84.1% sensitive and 98.9% specific when it was compared with the cytotoxin assay. The use of toxigenic culture to referee discrepant results (EIA versus cytotoxin assay) showed the EIA sensitivity and specificity to be 95.1 and 99.3%, respectively, with respect to other laboratory methods. Patient charts were reviewed for antibiotic-associated diarrhea on 108 specimens, including all those that were positive by at least one test method. Of 34 patients determined to have *C. difficile*-associated disease, 29 (85.3%) were positive by EIA, 32 (94.1%) were positive by the cytotoxin assay, 27 (79.4%) were positive by toxigenic culture, and 20 (58.8%) were positive by latex agglutination. Seven patients with antibiotic-associated diarrhea had a positive latex result, but results were negative by EIA, the cytotoxin assay, and toxigenic culture. The EIA demonstrated high specificity and good sensitivity for *C. difficile*-associated disease cases. The test can be used alone or in combination with the cytotoxin assay or toxigenic culture to provide rapid and sensitive results.

Clostridium difficile is the major cause of diarrhea among hospitalized patients, and its role in nosocomial or institutional antibiotic-associated diarrhea (AAD) has been extensively studied and reviewed (1, 2, 5, 6, 12, 15). Approximately 15 to 25% of AAD cases are caused by *C. difficile*. Evidence suggests that the nosocomial spread of *C. difficile* can occur through direct patient-to-patient contact, transmission to patients from the hands of hospital personnel, or acquisition of organisms from the environment. Carrier rates for *C. difficile* in hospitalized patients have been reported to be as high as 20% (20). The majority of those harboring the organism remain asymptomatic, but for patients who develop symptoms, the spectrum of disease ranges from self-limiting diarrhea to acute colitis and life-threatening megacolon. Most strains of *C. difficile* produce both an enterotoxin (toxin A) and a cytotoxin (toxin B). In animal models, toxin A has been linked with disease (4, 13) and may therefore be the most important marker for disease in humans (12).

Diagnostic testing in cases of suspected *C. difficile*-associated diarrhea can be accomplished by recovering the organism in culture, by demonstrating the presence of toxin B in stool by a tissue culture assay, or by performing a rapid latex agglutination test for *C. difficile*-associated antigen on stool specimens. These tests can be used alone or in combination, as is frequently recommended (7, 17, 18). Laboratory findings must be combined with clinical findings of AAD to diagnose *C. difficile*-associated disease (CDAD).

Purification of toxin A and the subsequent production of both polyclonal and monoclonal antibodies have led to the development of enzyme immunoassays (EIAs) which detect toxin A in stool specimens (14, 21, 22). EIA procedures provide rapid and accurate tests which are technically less complex to perform than either cytotoxicity assays or culture. In this report we describe the development of a new commercial EIA which detects toxin A and evaluate its use in the diagnosis of CDAD. Fresh stool specimens submitted for analysis were cultured for *C. difficile* and were tested by latex agglutination and for the presence of both toxins A and B. Patient histories were reviewed in an attempt to equate test results with the likelihood of AAD and CDAD.

MATERIALS AND METHODS

Bacterial strains. Toxigenic strains of *C. difficile* (VPI 10463, VPI 26689, VPI 7698, VPI 24040, VPI 11011, VPI 4474), a nontoxigenic strain of *C. difficile* (VPI 11186), and *Clostridium sordellii* (VPI 9048) were obtained from the Anaerobe Research Center Virginia Polytechnic Institute (VPI), Blacksburg. The following organisms were obtained from the American Type Culture Collection (ATCC), Rockville, Md.: *Aeromonas hydrophila* ATCC 7965, *Bacillus cereus* ATCC 14579, *Bacillus subtilis* ATCC 6051, *Bacteroides fragilis* ATCC 23745, *Candida albicans* ATCC 10231, *Clostridium botulinum* type E ATCC 17786, *Clostridium butyricum* ATCC 4855, *Clostridium histolyticum* ATCC

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19401, *Clostridium novyi* ATCC 19402, *Clostridium perfringens* ATCC 9714, *Clostridium sporogenes* ATCC 3584, *Clostridium subterminale* ATCC 25774, *Clostridium tetani* ATCC 10779, *Peptostreptococcus anaerobius* ATCC 27337, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* Cowan I ATCC 12598, *Vibrio cholerae* ATCC 14035, and *Vibrio parahemolyticus* ATCC 17802. The following organisms were obtained from Meridian Diagnostics, Inc.: *Escherichia coli*, *Salmonella* group B, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, *Streptococcus faecalis*, and *Yersinia enterocolitica*.

Specificity testing. Organisms were grown in brain heart infusion (BHI) broth (Difco, Detroit, Mich.) to $\geq 10^7$ organisms per ml. Known toxin A-positive and -negative stool specimens were diluted 1:9 in sample diluent and was then spiked with 25 μl of inoculated or uninoculated BHI broth. The samples were then tested by EIA as described below.

Western blots (immunoblots). A crude dialysis bag culture filtrate was prepared by growing toxigenic *C. difficile* VPI 10463 under anaerobic conditions within a saline-filled dialysis bag. The inoculated bag was suspended in BHI broth containing 0.1% agar for 72 h and was then filtered through a 0.45-μm-pore-size membrane (19). The filtrate was diluted 1:4 in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) reducing sample buffer and was heated to 95°C for 5 min in a sealed tube. The reduced sample was electrophoresed on a 1-mm 7.5% separating gel with a 4% stacking gel in a batch well for 2 h at 50 mA per gel. Following SDS-PAGE, the gel was soaked for 15 min in glycine-buffered methanol (pH 8.3), and the proteins were transferred to a membrane (nitrocellulose; MSI, Westboro, Mass.) by Western blotting (2 h, 150 mA). Membranes were blocked overnight (phosphate-buffered saline [PBS]–7% Carnation evaporated milk) and were probed with either horseradish-peroxidase-conjugated monoclonal detection reagent antibody (diluted 1:3 in PBS–3% bovine serum albumin [BSA]) or goat anti-*C. difficile* (diluted in 1:500 PBS–3% BSA; Virginia Polytechnic Institute) for 1 h. Goat anti-*C. difficile* lanes were washed four times (10 min each time) with PBS–0.3% BSA and were then probed with donkey anti-goat immunoglobulin G (heavy and light chain)–horseradish peroxidase conjugated (Jackson Immunoresearch, West Grove, Pa.) diluted 1:2,000 in PBS–3% BSA for 1 h. All blots were then washed three times with PBS–0.3% BSA and twice with PBS. Immunoreactive bands were developed by using hydrogen peroxide and the precipitating chromogen 4-chloro-1-naphthol (Kirkegaard and Perry, Gaithersburg, Md.).

Toxin A and B standard curves. Purified toxins (A and B) were kindly provided by David Lyerly (Virginia Polytechnic Institute). Toxins were diluted in buffer (1 part 0.01 M PBS–0.1% BSA, 0.05% Tween 20, 0.01% thimerosal [pH 7.2]; 4 parts sample diluent) and tested by the EIA as described below.

Toxin A adsorption studies were performed by using sheep (Kroy Medical, Stillwater, Mn.) and rabbit (Pel-Freez, Rogers, Ark.) erythrocytes. Erythrocytes were washed with PBS prior to use. Purified toxins A and B were adsorbed with sheep and rabbit erythrocytes at 4°C for 1 h and centrifuged, and the supernatants were tested by the EIA as described below.

Patient specimens. A total of 328 fresh stool specimens submitted to the Microbiology Laboratory at The Christ Hospital over an 8-month period for routine CDAD testing were evaluated. Specimens were held at 2 to 8°C prior to testing. Cytotoxin assays were performed each day. Toxigenic culture of *C. difficile* and the EIA for toxin A were performed on batched stool specimens that were refrigerated for up to 72 h. Residual specimen was frozen at −70°C for later evaluation if necessary.

Culture. A 1.0-ml portion of each stool sample was heated to 70°C for 10 min to kill non-spore-forming organisms. A 0.1-ml amount was then streaked for isolation onto cefoxitin-cycloserine-fructose agar (CCFA; Remel, Lenexa, Kans.) and was incubated anaerobically at 35°C for 48 h. Plates were examined for morphologically suspicious, fructose-positive (yellow) colonies. Suspicious colonies were confirmed by long-wave fluorescence and by use of biochemical tests (Rapid Ana II System; Innovative Diagnostic Systems, Inc., Atlanta, Ga.). Specimens which were positive by either EIA or cytotoxin but culture negative were recultured from frozen aliquots of stool (−70°C) onto CCFA (Carr-Scarborough, Decatur, Ga.) without heat shock. *C. difficile* isolates were grown in BHI broth for subsequent toxicity studies. All *C. difficile* isolates were tested for the in vitro production of toxins A and B by both the EIA and cytotoxin assays. Isolates that produced both toxin A (by EIA) and cytotoxin were classified as toxigenic. Isolates that produced neither toxin were classified as nontoxigenic. No isolates that produced only one toxin were found in this study. Only toxigenic isolates of *C. difficile* were considered to be etiological agents of CDAD in this study.

Latex agglutination. Specimens were tested by using the Culturette Brand CDT latex test (Becton Dickinson Microbiology Systems, Cockeysville, Md.). Stool (0.5 g) was mixed with buffer (0.5 ml), vortexed, and centrifuged at 1,500 × *g* for 15 min. One drop of supernatant was tested according to the instructions of the manufacturer. Nonspecific reactions (i.e., agglutination with both the antitoxin-coated and negative control latex particles) were reported as indeterminate.

Toxin B assay. Cytotoxin was detected in stool samples by using the Toxi-titer microtiter plate system (Bartels Immunodiagnostic Supplies, Inc., Bellevue, Wash.). Stool filtrates (final dilution, 1:40) were inoculated and read according to the instructions of the manufacturer. Plates were examined at 24 and 48 h. A positive assay was defined as a minimum of 25% cell rounding that could be neutralized by antitoxin (23). In general, most positive specimens showed much more than 50% rounding. Samples that demonstrated cytotoxic effects in the presence of antitoxin were defined as indeterminate.

Toxin A assay. *C. difficile* toxin A was detected in stool samples by using the PREMIER-*C. difficile* Toxin A EIA (Meridian Diagnostics, Inc., Cincinnati, Ohio). This one-step direct EIA procedure uses plastic microwells coated with polyclonal antibody to toxin A.

(i) Sample preparation. Liquid and semisolid stools (50-μl portions) were added to 200 μl of diluent buffer, mixed, and vortexed for 15 s. Formed stools were processed by transferring a small "BB"-sized portion (diameter, 3 to 4 mm) into 200 μl of the same buffer. The stool was emulsified with a wooden applicator stick and was then vortexed for 15 s.

(ii) Test performance. Following equilibration at room temperature, the required number of microwells (one well for each specimen plus one positive and one negative control well for each batch) were removed from the protective pouch. Microwells were placed in a 96-microwell strip holder, and one drop of enzyme conjugate (50 μl) was added to all wells. The diluted stool samples (50 μl) were then added to appropriate wells. Following the addition of positive and negative controls to the designated wells, the plate was mixed by gently shaking it for 30 s. Plates were sealed

and incubated for 2 h at 35°C. All microwells were washed five times with a squirt bottle containing wash buffer. After removal of the final wash buffer, one free-falling drop of substrate A was added to each well; this was followed by the addition of a drop of substrate B. The plate was gently shaken for 15 s to mix the substrates and was then incubated for 10 min at room temperature. One drop of stop solution was added to all wells, and the plate was gently shaken for 15 s to mix the substrates. The initial color of a positive reaction was blue; this changed to yellow upon the addition of the stop solution.

(iii) Reading. Plates were read visually within 15 min of the addition of the stop solution. Plates were read within 30 min by using an EIA reader (450 nm) zeroed on air. A reading of <0.100 was negative, a reading of ≥0.100 but <0.150 was indeterminate, and a reading of ≥0.150 was positive.

Clinical evaluations. An attempt was made to review the charts of all patients with specimens that were positive by one or more laboratory tests (EIA, cytotoxin assay, toxigenic culture, and latex agglutination). A number of randomly selected charts of patients whose specimens were negative for all four tests were also reviewed. Patient charts were evaluated for the likelihood of AAD. Patients were considered AAD positive if they had (i) a history of antibiotic administration within 8 weeks of the stool sample and (ii) six or more unformed or diarrheal stools over a 36-h period (18). Patients were also evaluated for other causes of diarrhea and their responses to specific therapy for *C. difficile*. The results from three toxin-dependent laboratory tests (EIA, cytotoxin assay, and toxigenic culture) were used in conjunction with chart review evidence of AAD to establish a likely diagnosis of CDAD for each case reviewed. Latex testing was not used to define CDAD because of the known cross-reactions with non-*C. difficile* isolates and the inability to distinguish toxigenic from nontoxigenic isolates. Patients with AAD and one or more positive test result (excluding latex agglutination) were classified as CDAD positive. Patients with AAD but whose stools were negative by EIA, cytotoxin, and toxigenic culture were classified as CDAD negative. All AAD-negative patients were classified as CDAD negative, regardless of the test results.

RESULTS

Toxin A specificity studies. The new toxin A EIA uses a polyclonal capture antibody to toxin A. The specificity of the test for toxin A is largely due to the use of a monoclonal detection antibody (13) conjugated to horseradish peroxidase. Only one immunoreactive band was observed with the detection antibody on Western blots of reduced SDS-polyacrylamide gels containing crude culture filtrate (Fig. 1). The many other antigens present in the blots were visualized with goat anti-*C. difficile* 10463. The identity of the band observed with the monoclonal detection antibody was verified by spiking crude culture filtrate with highly purified toxin A (single arc on crossed immunoelectrophoresis; data not shown). The monoclonal antibody-reactive band was enhanced in the toxin A-spiked lane. The mobility of this band corresponded to that of toxins A and B. Toxins A and B have similar molecular weights and can comigrate by SDS-PAGE (12, 19). The EIA was tested over a broad range by using purified toxins A and B (Fig. 2). The assay was at least 5,000 times more sensitive for toxin A than it was for toxin B. The assay was linear from 20 to at least 250 pg (2 to 25 ng of toxin A per ml in the original specimen). Higher levels of toxin A were off scale.

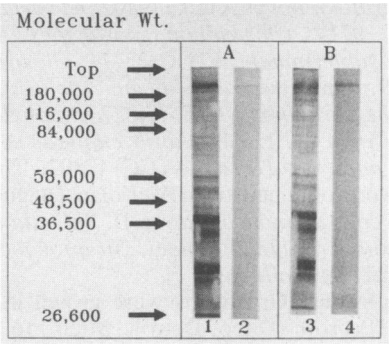

FIG. 1. Specificity of EIA detection antibody versus Crude *C. difficile* culture filtrate. Crude *C. difficile* VPI 10463 culture filtrate (100 μl per gel) containing 54 μg of toxin A per ml (A) or spiked with an additional 100 μg of toxin A per ml (B) was run on 7.5% gels. Gels were probed with either goat anti-*C. difficile* (lanes 1 and 3) or monoclonal detection antibody (lanes 2 and 4).

Toxin A, but not toxin B, has been shown to bind to rabbit erythrocytes (3, 9). Neither toxin binds to sheep erythrocytes (11). Purified toxin B was adsorbed with rabbit erythrocytes in an attempt to remove any contaminating toxin A. Eighty-one percent of the toxin B absorbance remained after adsorption with rabbit erythrocytes. The toxin A absorbance was reduced by 94% upon adsorption with rabbit erythrocytes. These results indicated cross-reaction with toxin B, but at 5,000-fold higher levels. Alternatively, toxin A impurities present in the toxin B preparation may have lost their binding capacity during purification or storage.

The specificity of the EIA was evaluated by spiking positive and negative stool specimens with high (≥10^7 to 10^9 CFU/ml) levels of various microorganisms. Of the 28 microorganisms tested, 27 gave negative results when the microorganisms were added to negative stool specimens. Similarly, the identical organisms did not give false-negative results in positive stool specimens. An exception was a highly toxigenic isolate of *C. sordellii* (VPI 9048), which was

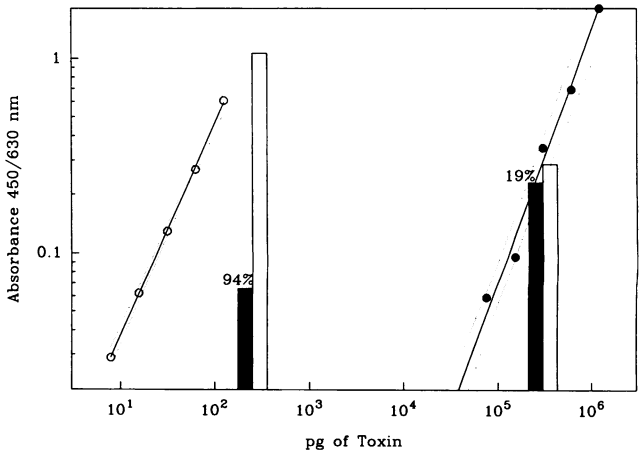

FIG. 2. EIA sensitivity and specificity for purified toxins A and B. Purified toxins A (open circles) and B (closed circles) were tested in the EIA as described in the text. Vertical bars show rabbit (solid bars) and sheep (open bars) erythrocyte adsorptions of 250 pg of toxin A and 300 ng of toxin B. Dotted lines represent 99% confidence limits of linear regression lines. Log scales are used because of the extraordinarily large range covered on the *x* axis (5 log units).

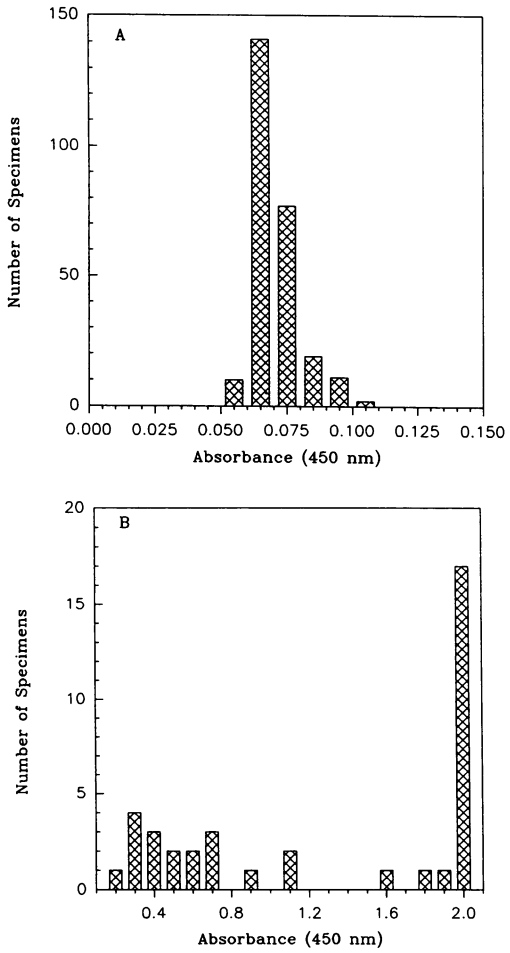

FIG. 3. Observed frequencies of EIA results with clinical specimens. The A_{450} for all EIA-negative and indeterminate specimens (A) and all EIA-positive specimens (B) were grouped within appropriate absorbance ranges and plotted as histograms.

positive in the EIA. A less toxic isolate of *C. sordellii* (ATCC 9714) was negative in the EIA. A panel of *C. difficile* reference strains (Virginia Polytechnic Institute) ranging from highly toxigenic to negative was tested by the EIA. All of the toxigenic strains (n = 6) were positive, and the nontoxigenic strain (VPI 11186) was negative (data not shown). In addition, 46 toxigenic clinical isolates from this study were positive for both toxin A and B production in vitro. Another six isolates which did not produce toxin B in vitro were also negative by the EIA.

Toxin A EIA positive and negative distributions. The EIA results for positive and negative patient specimens are shown in Fig. 3. The negative (A_{450} <0.100) and indeterminate (0.100 ≤ A_{450} <0.150) specimens had a mean absorbance of 0.066 ± 0.011 (standard deviation) (Fig. 3A). Slight positive skewing and kurtosis were observed relative to the normal distribution. The positive cutoff level (A_{450} = 0.150) was 7.6 standard deviation units above the mean of the negative specimens, which gave essentially a zero probability that a known toxin A-negative specimen would be read as a false-positive specimen.

The EIA-positive specimens were neither normally nor uniformly distributed (Fig. 3B). Therefore, statistics regarding the mean (1.257) and standard deviation (0.941) were not meaningful. It was found that 58% of the EIA-positive specimens gave high readings (A_{450}, ≥ 1.000); 29% were moderate (0.300 ≤ A_{450} < 1.000); and 13% were weak (0.150 ≤ A_{450} < 0.300). In addition, a loose relationship seemed to suggest that very diarrheal specimens (nearly clear liquid) were so dilute as to give weak to moderate readings.

TABLE 1. Comparison of EIA, cytotoxin assay, toxigenic culture, and latex agglutination testing by using 313 fresh clinical specimens

Result for[a]:			No. of specimens	No. of specimens with the following latex agglutination assay result:		
EIA	Cytotoxin	TCU		+	−	Ind[b]
+	+	+	33	22	9	2
+	+	−	4	3	1	0
+	−	+	1	1	0	0
−	+	+	2	2	0	0
+	−	−	2	0	1	1
−	+	−	5	0	5	0
−	−	+	7	0	7	0
−	−	−	259	8	234	17

[a] TCU, toxigenic culture; +, positive test; −, negative test.
[b] Ind, indeterminate.

Clinical evaluation. Specimens (n = 328) submitted for the diagnosis of CDAD were tested by four laboratory methods (EIA, cytotoxin assay, toxigenic culture, and latex agglutination). The comparative results (excluding indeterminate results) are given in Table 1. Sixty-two (19.8%) were positive by at least one method, but only 22 (7.0%) were positive by all four methods. The number of positive tests for each method were EIA, 40 (12.8%); cytotoxin assay, 44 (14.1%); toxigenic culture, 43 (13.7%); and latex agglutination, 36 (11.5%). Nontoxigenic isolates of *C. difficile* were recovered from six specimens. Forty specimens (12.8%) were positive by two or more methods, while 22 specimens (7.0%) were positive by only one laboratory test. The results of EIA agreed with the results of the cytotoxin assay, toxigenic culture, and latex agglutination for 303 (96.8%), 298 (95.2%), and 272 (86.9%) specimens, respectively.

A comparison of the EIA with the cytotoxin assay is given in Table 2. Indeterminate results by both tests are included. The EIA was 84.1% sensitive and 98.9% specific relative to the cytotoxin assay. The sensitivity of the EIA increased to 95.1% when toxigenic culture was used to referee discrepant results. One of three cytotoxin-negative, EIA-positive specimens and the one cytotoxin indeterminate, EIA-positive

TABLE 2. Comparison of EIA and cytotoxin assays by using toxigenic culture to resolve discrepant results

EIA	No. of specimens with the following results for[a]:					
	Cytotoxin			Cytotoxin/TCU[b]		
	+	−	±	+	−	±
+	37	3	1	39	2	0
−	7	266	1	2	272	0
±	2	3	0	1	4	0

[a] +, positive test; −, negative test; ±, indeterminate test. The sensitivity, specificity, and correlation for the cytotoxin and cytotoxin/TCU assays were 84.1 and 95.1%, 98.9 and 99.3%, and 96.8 and 98.7%, respectively.
[b] Cytotoxin/TCU, discrepant results between EIA and the cytotoxin assay refereed by using toxigenic culture.

TABLE 3. Comparison of laboratory methods for detection of *C. difficile* in patients with AAD and CDAD

No. of charts reviewed/ total no.[a]	Test result			No. of the following patients with the indicated response to treatment:						CDAD[c]		
				AAD positive			AAD negative					
	EIA	Cytotoxin	TCU[b]	+	−	NT[d]	+	−	NT	+	−	Follow-up
29/33	+	+	+	23	0	4	1	0	1	25 (17)	2 (1)	2 (1)
3/4	+	+	−	2	0	1	0	0	0	3 (2)	0 (0)	0 (0)
1/1	+	−	+	0	0	0	0	0	1	0 (0)	1 (0)	0 (0)
2/2	−	+	+	1	0	0	0	0	1	1 (1)	1 (1)	0 (0)
2/2	+	−	−	0	0	1	0	0	1	1 (0)	1 (0)	0 (0)
5/5	−	+	−	4	0	0	0	0	1	3 (0)	1 (0)	1 (0)
7/7	−	−	+	3	1	2	0	0	1	1 (0)	1 (0)	5 (0)
59/259	−	−	−	5	2	20	1	0	31	0 (0)	250 (8)	9 (0)
108/313				38	3	28	2	0	37	34 (20)	257 (10)	17 (1)

[a] Five charts were not available for review.
[b] TCU, culture for toxigenic *C. difficile*.
[c] CDAD was defined as AAD positive with one or more positive laboratory tests for *C. difficile* (EIA, cytotoxin assay, or toxigenic culture). Follow-up refers to specimens obtained several days after an initial positive stool. The number of patient specimens with positive latex agglutination results are given in parentheses.
[d] NT, the patient was not treated.

specimen were culture positive for toxigenic *C. difficile*. In addition, one of two cytotoxin-positive, EIA-indeterminate; five of seven cytotoxin-positive, EIA-negative; and the one cytotoxin-indeterminate, EIA-negative specimens were culture negative for toxigenic *C. difficile*, which increased the relative specificity of the EIA to 99.3%.

The comparisons shown in Table 2 give a measure of the value of the toxin A EIA relative to cytotoxin testing. However, determination of the diagnostic value of each method for patient disease is a more critical measure of test efficacy. For this reason, chart reviews for AAD were conducted for 108 patient specimens, including 57 of 62 specimens positive by at least one laboratory test. The results of three toxin-dependent laboratory tests (EIA, cytotoxin assay, and toxigenic culture) were used in conjunction with chart review evidence of AAD to establish a likely diagnosis of CDAD for each case reviewed (Table 3). Results of latex agglutination testing were not used to determine CDAD. The latex agglutination-reactive protein is not related to or dependent upon *C. difficile* toxin production and is not species specific. However, latex agglutination results were included for comparison with CDAD and other methods. Of 33 specimens that were positive by all three tests, 29 were reviewed for evidence of AAD and response to specific treatment for CDAD. All treated AAD-positive patients (23 of 23) responded to specific therapy. However, only 18 of 27 (67%) specimens from patients in this group were latex agglutination positive. Two specimens that were positive by all three methods were from patients with diarrhea but for whom documentation of antibiotic history was incomplete. These specimens were classified as AAD negative and, thus, CDAD negative. One of these specimens was latex agglutination positive and was from a patient who was treated and who responded to therapy. The patient (age, 62 years; female) had nephrotic syndrome with chronic renal failure, achalasia, and a previous episode of CDAD (i.e., a relapse patient). The other specimen was latex agglutination indeterminate, and the patient from whom the specimen was obtained was not treated. This patient was a 45-year-old female surgical inpatient (4 days) with a small-bowel obstruction.

Chart review results for patients with specimens that were positive by one and two laboratory tests are also presented in Table 3. Of 14 patients in this group, 9 were determined to have CDAD. Only three of the nine were latex agglutination positive. Five patients in this group were CDAD negative. One patient (pregnant female; age, 28 years; EIA and toxigenic culture positive) had *Salmonella typhi* sepsis and *Shigella sonnei* in her stool. While the patient had diarrhea, no previous antibiotic treatments were recorded. Another patient (age, 85 years; female; cytotoxin and toxigenic culture positive) was treated with antibiotics but had no clinically significant diarrhea prior to the date the specimen was obtained (inpatient for 19 days). This patient had an acute subdural hematoma and was transferred before the results of vancomycin therapy could be documented. The CDAD-negative patient with EIA as the only positive test had Down's syndrome (age, 34 years; male) and was treated with antibiotics with no documentation of diarrhea. The cytotoxin-positive, CDAD-negative patient (age, 45 years; male; inpatient for 3 days) was diabetic with cholestatic hepatitis and pancreatic insufficiency. The patient had no antibiotic treatments prior to the date the specimen was obtained but had severe diarrhea. The patient was not treated for *C. difficile* disease. Finally, the toxigenic culture-positive, CDAD-negative patient (age, 31 years; male) had chronic renal failure, Barretts esophagus, and a hiatal hernia. No prior antibiotic or diarrheal history was noted.

Fifty-nine specimens that were negative by all three toxin-dependent laboratory tests were also studied. Twenty-seven specimens came from patients with evidence of AAD. Five of the seven treated patients responded to therapy. Of the 250 specimens from CDAD-negative patients, 8 were latex agglutination positive. Seven of eight latex agglutination-positive specimens were from AAD-positive patients (the chart of one patient was not available). Nontoxigenic isolates of *C. difficile* were isolated from two of these eight specimens. In addition, one specimen which was EIA and cytotoxin positive yielded only nontoxigenic *C. difficile*, even when multiple colonies were sampled. This observation was made twice.

The correlation of each laboratory method with AAD and CDAD is shown in Table 4. The correlation of each test with AAD was low, ranging from 59.3% (latex agglutination) to 64.8% (cytotoxin assay). Correlations with CDAD were higher, ranging from 91.8% (latex agglutination) to 97.9%

TABLE 4. Correlation of laboratory methods for *C. difficile* with AAD and CDAD

Test method	% Correlation[a]	
	AAD (n = 107)	CDAD (n = 290)
EIA	61.1	96.9
Cytotoxin assay	64.8	97.9
Toxigenic culture	63.0	95.9
Latex agglutination	59.3	91.8

[a] Correlation = (number of true positive patients + number of true negative patients/total number of patients.

(cytotoxin assay). Performance statistics related to CDAD are given in Table 5. The cytotoxin assay was the most sensitive test (94.1%), and latex agglutination was the least sensitive (58.8%).

DISCUSSION

C. difficile causes diarrhea and colitis in hospitalized adults, primarily those receiving antibiotics such as clindamycin, ampicillin, or cephalosporins (5). Approximately 10 to 20% of the stool specimens submitted to the laboratory for the diagnosis of CDAD are positive for cytotoxin (21). The more serious the disease the more likely it is that *C. difficile* toxin B will be detected in specimens. Some specimens positive by laboratory tests are from patients who have no documentation of antibiotic treatment. Such positive test results may reflect asymptomatic carriage of *C. difficile* (15). The most appropriate test or group of tests to use for the diagnosis of CDAD remains controversial. The tissue culture cytotoxin assay is considered by some to be the diagnostic test of choice. The assay is able to detect as little as 1 pg of toxin B. Problems with the test procedure include a lack of standardization, the need for tissue culture capabilities, the high cost, and the fact that results are not available for 24 to 48 h. Isolation of *C. difficile* on CCFA has also been used. The method was shown in several studies to be more sensitive than but not as specific as cytotoxin testing (16, 17). Culture also takes at least 24 to 48 h to complete, and some isolates of *C. difficile* do not demonstrate toxin production. In addition, various culture methods exist, and no clear standard has been chosen. The latex agglutination test is rapid and easy to perform, but the test detects an antigen that is unrelated to toxin. Nontoxigenic strains of *C. difficile* as well as other organisms that produce this antigen react with latex. It is advocated by some groups that at least two different tests be performed in order to provide maximum sensitivity.

The diagnosis of CDAD remains problematic. The presence of diarrhea with a history of recent or concomitant antimicrobial therapy is only suggestive of disease. Laboratory tests must be used to aid in the diagnosis. In this study, only 61% of AAD-positive patients had laboratory evidence of *C. difficile*. *C. difficile* is often recovered from the stools of patients with AAD, but asymptomatic carriage is prevalent in certain patient populations (5). False-positive as well as false-negative cytotoxin assays are also possible. In previous studies, culture was shown to be the most sensitive and least specific test for CDAD, while the cytotoxin assay was the least sensitive and most specific test for this disease (16, 17).

TABLE 5. Performance of laboratory methods for diagnosis of CDAD

Test method	Sensitivity (%)	Specificity (%)	PV (%)[a]	
			+	−
EIA	85.3	98.4	87.4	98.1
Cytotoxin assay	94.1	98.4	88.9	99.2
Toxigenic culture	79.4	98.1	84.4	97.3
Latex agglutination	58.8	96.1	66.7	94.6

[a] PV, predictive value.

The production of both polyclonal and monoclonal antibodies to purified *C. difficile* toxin A has permitted the development of a microwell EIA that can detect toxin A in stool specimens. In this study, a new EIA was compared with standard tests for the detection of CDAD. Patient charts were reviewed for evidence of AAD and CDAD, and the findings were used to determine the diagnostic accuracy of each laboratory test. Stool specimens from patients with pseudomembranous colitis generally have cytotoxin titers of $\geq 10^2$, and these levels of toxin B correspond to toxin A concentrations ≥ 1 ng/ml (12). It has therefore been suggested that any assay for toxin A should have a sensitivity approaching 1 ng/ml (12). The new EIA described here can detect approximately 2 ng of toxin A per ml. Earlier EIAs for toxin A approached this sensitivity level, but they took longer and required time-consuming specimen centrifugation and filtration steps (8, 10). The EIA was specific for toxin A, the only significant cross-reaction being with one strain of *C. sordellii*, which is also positive in most cytotoxin assays.

Clinical results showed that the cytotoxin assay is the most sensitive test (94.1%) for the diagnosis of CDAD, followed by EIA (85.3%), toxigenic culture (79.4%), and latex agglutination (58.8%). The sensitivity of toxigenic culture in this study was lower than that reported for culture by others (6, 16, 17). This may be due to the screening of *C. difficile* isolates for in vitro toxin production in this study, thus eliminating six specimens that yielded nontoxigenic isolates. The total incidence of *C. difficile* isolates in this study (52 isolates from 328 patient specimens; 15.9%) was consistent with previous reports (16, 17). Forty-six of these isolates (88.5%) were toxigenic (i.e., produced both toxin A, as determined by EIA, and cytotoxin in vitro) strains of *C. difficile*. None of the six nontoxigenic isolates produced either toxin A or cytotoxin in vitro. Latex agglutination demonstrated lower sensitivity because the test was negative for 8 of 26 AAD-positive specimens that were positive for all other laboratory tests. The specificities of EIA, the cytotoxin assay, and toxigenic culture were similar (98.4, 98.4, and 98.1%, respectively). A lower specificity for latex agglutination (96.1%) was seen because eight latex agglutination-positive specimens were negative by all other laboratory tests. However, seven of these specimens were from AAD-positive patients. Two of these patients were found to harbor nontoxigenic *C. difficile* strains. We realized that the case definition of CDAD can influence the performance statistics of individual tests. The results in this study are presented in a manner that permits other comparisons and interpretations.

The EIA demonstrates excellent specificity and good sensitivity compared with those of the cytotoxin assay and toxigenic culture. Although EIA is not as rapid as latex agglutination, the test can be performed in several hours, is less technically demanding than the cytotoxin assay, and is suitable for batch runs. The EIA can be used alone or in combination with other methods (cytotoxin assay or culture) to provide rapid and sensitive results.

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