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Comparison of Three Enzyme Immunoassays, a Cytotoxicity Assay, and Toxigenic Culture for Diagnosis of *Clostridium difficile*-Associated Diarrhea

FRÉDÉRIC BARBUT,* CHRISTOPHE KAJZER, NADINE PLANAS, AND JEAN-CLAUDE PETIT

Service de Bactériologie-Virologie, Hôpital Saint-Antoine, Paris, France

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Enzyme immunoassays (EIAs) based on monoclonal antibodies for the detection of *Clostridium difficile* toxins have recently been developed for clinical use. The aim of this study was to compare three commercially available EIAs, two for toxin A (Premier *C. difficile* Toxin A; Meridian, Osi, Elancourt, France; and Vidas *C. difficile* Toxin A; bioMérieux, Marcy l'Etoile, France) and one for toxins A and B (Cytoclone A + B EIA; Cambridge Biotech Corp., Codiapharm, Evian, France), with a cytotoxicity assay and toxigenic culture for the diagnosis of *C. difficile*-associated diarrhea (CDAD). The study was performed with 285 fresh stools from 285 patients with suspected CDAD. In case of disagreement, the tests were repeated on a frozen aliquot of the same stool sample, and the patient's chart was reviewed. CDAD diagnosis was established in 55 cases (incidence, 19.3%). The sensitivities and specificities of the methods were, respectively, 92.7 and 100% for the cytotoxicity assay, 96.4 and 99.1% for toxigenic culture, 75.5 and 97.8% for Cytoclone, 65.4 and 99.6% for Premier, and 65.4 and 100% for Vidas. The results were uninterpretable in 3.2% of cases with Cytoclone, 0.3% with Premier, and 2.5% with Vidas. We conclude that the cytotoxicity assay and toxigenic culture remain the best methods for the diagnosis of CDAD even though they lack standardization and require 48 to 96 h to obtain the result. Despite their rapidity and simplicity, EIAs are not sensitive enough to be relied on as the sole laboratory test.

Clostridium difficile is a major cause of gastrointestinal disorders among hospitalized patients: this anaerobic bacterium is responsible for 20 to 25% of cases of antibiotic-associated diarrhea and/or colitis and for 90% of cases of pseudomembranous colitis (3, 4, 17). *C. difficile* infections can be acquired through direct patient-to-patient contact and from the hospital environment (14, 22, 23); such nosocomial transmission can contribute significantly to the length of hospital stay.

Most strains of *C. difficile* produce two toxins, A and B, which act in synergy on the intestinal mucosa (3, 32). Toxin A is an enterotoxin that causes hemorrhagic fluid accumulation in rabbit ileal loops. Toxin B, which lacks enterotoxic activity, is a highly potent cytotoxin for most cell lines.

Diagnostic methods for *C. difficile*-associated diarrhea (CDAD) are based on isolation of the organism, detection of cell wall antigens by latex agglutination methods, or detection of toxins in stool specimens (for reviews, see references 6, 17, and 25). So far, the cytotoxicity assay (tissue culture assay) for toxin B has been considered the "gold standard," although it requires experience in tissue culture techniques and up to 48 h of incubation. Recently, several enzyme immunoassays (EIAs) based on monoclonal antibodies against toxin A and/or B have become available (2, 8–10, 27). The aim of this study was to evaluate and compare two EIAs detecting toxin A (Premier *C. difficile* Toxin A; Meridian, Osi; Vidas *C. difficile* Toxin A; bioMérieux) and one EIA detecting both toxin A and toxin B (Cytoclone A + B EIA; Cambridge Biotech Corp., Codiapharm) for the diagnosis of CDAD. The tests were performed in parallel with the tissue culture assay and toxigenic culture of fecal specimens.

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* Corresponding author.

MATERIALS AND METHODS

Specimens. Two hundred eighty-five stool specimens from 285 patients suspected of having CDAD were submitted to the laboratory of Saint-Antoine Hospital over a 4-month period in 1992. All specimens were maintained at 4°C and processed within 72 h of collection; an aliquot of each specimen was frozen at −80°C in case further studies were required.

Culture. Stool specimens were inoculated onto a selective medium for *C. difficile* (brain-heart infusion supplemented with 5% defibrinated horse blood, 0.1% sodium taurocholate, 250 mg of cycloserine per liter, and 10 mg of cefoxitin per liter) (11, 33) and incubated at 37°C for 48 h in an anaerobic atmosphere. To ensure quality control of our medium, *C. difficile* reference strains (ATCC 43594, ATCC 43596, and ATCC 43598, generously provided by V. Delmee) were tested in each new batch. The identification of suspect colonies (based on characteristic morphology, Gram staining, and odor) was confirmed by using biochemical tests (Rapid ID 32A and API; bioMérieux, La Balme-les-Grottes, France) (5, 13).

Stool cytotoxicity assay. Fresh stool specimens were diluted in phosphate-buffered saline (1:10, wt/vol) and centrifuged at 2,500 × *g* for 30 min. The supernatant was passed through a 0.45-μm-pore-size filter and inoculated onto confluent monolayers of MRC-5 cells in 96-well microtiter plates that were incubated at 37°C in a 6.5% CO_2 atmosphere for 48 h (7). Screening for cytotoxicity was performed at stool dilutions of 1:100. Samples were considered positive in the cytotoxicity assay if a characteristic cytopathic effect (cell rounding) was observed and could be neutralized with

anti-*Clostridium sordellii* antiserum (obtained from M. Sebald, the Anaerobes Unit, Institut Pasteur, Paris, France). Neutralization was performed at a final dilution of 1:200.

EIAs. The Cytoclone assay is an EIA for the direct detection of *C. difficile* toxins A and B with two monoclonal anti-toxin A and anti-toxin B antibodies coated on wells of microtiter trays. Stool specimens are diluted 1:5 in diluent buffer, vortexed, and centrifuged at 2,500 $\times$ g for 15 min. Aliquots of supernatant (100 μl) are placed in each microwell and incubated for 2 h at room temperature. Positive and negative controls are included with each assay run to check the quality of the reagents and procedures. After five washes, 100 μl of a biotinylated goat polyclonal conjugate specific for each toxin is added and incubated for 15 min at room temperature. Unbound biotinylated conjugate is removed by washing. A streptavidin-horseradish peroxidase conjugate (100 μl) is then added, and the mixture is incubated for 15 min at room temperature. After a final washing step, a substrate (urea peroxide) and a chromogen (tetramethylbenzidine) mixture are added to the wells. The reaction is stopped with sulfuric acid, and the intensity of coloration is read spectrophotometrically (450 nm) within 30 min. The absorbance values (optical densities [OD]) are interpreted according to the manufacturer's instructions as follows: OD < 0.200, negative; $0.2 \leq$ OD < 0.25, indeterminate; OD $\geq$ 0.25, positive.

The Premier assay is based on microtiter wells coated with a polyclonal antibody to toxin A (capture antibody) and an enzyme-conjugated monoclonal antibody to toxin A (detector antibody). The test was performed according to the manufacturer's instructions. A small portion of stool is diluted 1:5 in 200 μl of diluent buffer and vortexed; the diluted stool sample (50 μl) is added to microwells with 1 drop of enzyme conjugate (monoclonal antitoxin A conjugated to horseradish peroxidase). Negative and positive controls are distributed in designated wells. The plates are sealed and incubated for 2 h at 37°C, and then the microwells are washed manually five times with wash buffer. One free-falling drop each of substrates A (urea peroxide) and B (tetramethylbenzidine) is added, and the mixture is incubated for 10 min at room temperature. The reaction is stopped with 1 drop of sulfuric acid, and coloration is read at 450 nm within 30 min of the addition of the stop solution. The absorbance values (OD) given by the manufacturer are as follows: OD < 0.100, negative; $0.100 \leq$ OD < 0.150, indeterminate; OD ≥ 0.150, positive.

The Vidas test is an automated enzyme-linked fluorescence assay for the detection of *C. difficile* toxin A. A pipette tip-like disposable device, the solid-phase receptacle (SPR), serves as the solid phase as well as the pipettor. The SPR is coated with a polyclonal rabbit anti-toxin A antibody to capture *C. difficile* toxin A specifically in stool specimens. The dual-reagent (test plus negative control) strips end with two optical cuvettes. This setup is still available in France, but it has been replaced by a single cuvette strip in the United States.

The Vidas test was performed according to the manufacturer's recommendations, except that the stool preparation step was slightly modified: stool specimens (0.5 g or 1 ml) were mixed with 1 ml of diluent buffer, vortexed, and centrifuged at 3,300 $\times$ g for 15 min, and supernatants were passed through 0.45-μm-pore-size filters. For this assay, the sample (300 μl) is pipetted into sample and reference wells of the dual-reagent strip and cycled automatically in and out of the SPR. After unbound sample components have been washed away, a mouse monoclonal anti-*C. difficile* toxin A antibody is introduced and cycled in and out of the SPR. After additional washes, an anti-mouse antibody conjugated to alkaline phosphatase is cycled in and out of the SPR. After a final wash step, a fluorescent substrate (4-methylumbelliferyl phosphate) is introduced into the SPR. Enzyme remaining on the wall of the SPR catalyzes conversion of the substrate to the fluorescent product 4-methylumbelliferone. The intensity of fluorescence is measured by the optical scanner in the Vidas apparatus. The reference side of the dual-reagent strip is treated identically except that instead of mouse anti-*C. difficile* toxin A antibody, it contains normal mouse serum. The sample reference strip is used to determine the amount of nonspecific background fluorescence generated by the specimen. The test value is calculated from fluorescence readings of the test sample and the reference sample strips. The results are analyzed automatically by the computer and interpreted as follows: $x < -300$, invalid; $-300 < x < 130$, negative; $130 \leq x < 235$, equivocal; $x \geq 235$, positive. Positive and negative controls are run on each new batch to ensure that assay performance has remained stable through storage.

Toxigenic culture. *C. difficile* strains isolated on selective media were tested for their production of toxins A and B in vitro (27). They were inoculated into prereduced trypticase-glucose-yeast broth and incubated anaerobically at 37°C for 5 days. The culture supernatant was assayed for toxin B in the same way as stool specimens (see above). Toxin A was tested for in the broth supernatant by using the Vidas test.

Analysis of discordant results. Stool specimens yielding discordant results in the stool cytotoxicity assay, toxigenic culture, and EIAs and those giving an indeterminate result were tested again by all five methods with the aliquots stored at −80°C.

Clinical assessment. Whenever the results of the five tests were not unanimous, the patients' charts were reviewed. Patients were considered to have had CDAD if they met five biological and clinical criteria (8): (i) diarrhea (three or more loose or watery stools a day for at least 2 days); (ii) antibiotic use within the 6 weeks preceding the onset of diarrhea; (iii) no other documented enteric pathogens; (iv) improvement of diarrhea after antibiotic withdrawal or response to oral vancomycin or metronidazole if administered; and (v) stool samples positive in toxigenic culture, cytotoxicity assay, or one EIA.

Analysis of test performance. Test performance was calculated in two ways. In one analysis, we compared the EIAs with the cytotoxicity assay, considered the gold standard for *C. difficile* toxins. We then evaluated the sensitivities, specificities, and positive and negative predictive values of each method on the basis of the CDAD case definition.

RESULTS

All 285 stool specimens were analyzed by the five methods. The results of repeat tests on frozen samples, when performed, were considered final. Of the 285 samples, 210 yielded negative results by the five methods and were considered true negatives; 32 samples were positive by all five methods and were considered true positives. The remaining 43 samples yielded indeterminate or discordant results even after repeat testing; Table 1 summarizes the interpretation of these cases on the basis of the patients' charts. Twenty-three cases were considered positive according to the biological and clinical criteria for CDAD listed

TABLE 1. Analysis of 43 samples yielding indeterminate or discrepant results after repeat testing

No. of cases	Results in[a]:					
	Cytotoxin assay	Cytoclone	Premier	Vidas	Toxigenic culture	CDAD cases
8	+	−	−	−	+	+
6	−	Ind	−	−	−	−
5	−	+	−	−	−	−
3	−	−	−	Inv	−	−
3	−	−	−	−	+	+
2	+	Ind	−	−	+	+
2	+	+	−	−	+	+
2	−	−	−	−	+	−
2	+	+	+	Equi	+	+
1	+	−	−	−	−	+
1	−	Ind	−	−	−	−
1	−	−	Ind	−	−	−
1	−	−	−	Equi	−	−
1	−	+	−	−	+	+
1	+	+	+	+	−	+
1	+	−	+	Inv	+	+
1	+	+	−	+	+	+
1	+	+	+	−	+	+
1	−	−	+	−	−	−

[a] Ind, indeterminate; Equi, equivocal; Inv, invalid.

above. The remaining 20 cases were considered negative. The overall incidence of CDAD was thus 19.3%.

Indeterminate results for initial specimens can affect the feasibility of tests in routine use. Cytoclone, Premier, and Vidas gave 6.7, 5.3, and 3.8% uninterpretable results with the initial samples. These figures fell to 3.2, 0.3, and 2.5% after repeat testing. Table 2 compares the results of repeat testing with the diagnosis of CDAD. Indeterminate results were excluded from the analysis of test performance, but the concordance between test results and CDAD diagnosis takes them into account. The degree of concordance was 98.6% for the cytotoxicity assay and toxigenic culture, 90.5% for Cytoclone, 92.6% for Premier, and 89.5% for Vidas.

The performance results for each method are given in Table 3. The cytotoxicity assay detected 51 of 55 true-positive CDAD cases and gave no false-positive results. Toxigenic culture missed two cases of CDAD and gave two false-positive results. Four toxigenic strains were isolated from patients who had negative cytotoxicity test and EIA results but who met the CDAD criteria. Thirteen false-negative or indeterminate results and five false-positive results were obtained with Cytoclone. Premier gave only one false-positive and one indeterminate result but failed to identify 19 true-positive specimens. Vidas had the best specificity but missed 18 cases of CDAD; of the 7 uninterpretable results, 4 were invalid and 3 were equivocal.

The performance of EIAs for detection of *C. difficile* toxins was also assessed by calculating their sensitivities, specificities, and predictive values relative to those of the cytotoxicity assay (Table 4). The results were similar to those obtained when the CDAD diagnostic criteria were used.

All the toxigenic *C. difficile* strains isolated on selective media, including those which gave negative results with the Vidas test directly applied to stools, produced both toxin A and toxin B in vitro. We also isolated 18 nontoxigenic strains.

DISCUSSION

The growing concern among physicians regarding enteric diseases associated with *C. difficile* has placed a heavy demand on clinical microbiology laboratories to offer a rapid and reliable diagnostic test. Methods proposed so far include counterimmunoelectrophoresis (26, 35), latex agglutination (15, 16, 20), dot immunobinding (34), and EIAs using polyclonal antiserum (1, 18, 19, 31), but all lack either sensitivity or specificity. The stool cytotoxicity assay is still considered the gold standard for *C. difficile* toxin B in stool specimens because of its high sensitivity (<1 pg of toxin B) and specificity (neutralization of the cytopathic effect by a specific antiserum) (7). Nevertheless, this method is time-consuming (up to 48 h of incubation), requires expertise in tissue culture techniques, and lacks standardization. The purification of toxins A and B has permitted the production of monoclonal antibodies against toxins A and B and the development of new and rapid immunoassays to detect these toxins in stool specimens (2, 8–10, 27).

In this study, we compared three commercial immunoassays (two based on monoclonal antibodies against toxin A [Premier and Vidas] and one against toxins A and B [Cytoclone]) with the cytotoxicity assay with 285 fresh stool specimens. As no single laboratory test is adequate for the diagnosis of CDAD, the patients' charts were reviewed and the findings were used to judge the diagnostic accuracy of each test.

The performance characteristics of the tests are listed in Table 3. The cytotoxicity test and toxigenic culture remained the most sensitive (>92%) and specific (>99%) methods. Nevertheless, the results of the cytotoxicity assay depend, to some extent, on the cell line, the age of the cells, the way in which stool specimens are processed, and the test format; moreover, they can sometimes be limited by interfering substances in the stool specimens (21, 28).

Toxigenic culture has the same limitations but requires

TABLE 2. Analysis of indeterminate results obtained with EIAs[a]

Cytoclone				Premier				Vidas			
No. of cases	1st	2nd	CDAD	No. of cases	1st	2nd	CDAD	No. of cases	1st	2nd	CDAD
5	I	−	−	10	I	−	−	2	Inv	−	−
5	I	−	+	3	I	−	+	3	Inv	Inv	−
7	I	I	−	1	I	+	+	1	Inv	Inv	+
2	I	I	+	1	I	I	−	3	Equi	−	−
								1	Equi	−	+
								1	Equi	Equi	−

[a] I, indeterminate; Inv, invalid; Equi, equivocal; 1st, result of initial testing; 2nd, result of repeat test of initial specimen; CDAD, diagnosis of CDAD.

TABLE 3. Characteristics of tests for diagnosis of CDAD[a]

Test	Sensitivity (%)	Specificity (%)	Predictive value		Correlation (%)
			Positive (%)	Negative (%)	
Cytotoxicity assay	92.7	100	100	98.3	98.6
Toxigenic culture	96.4	99.1	96.4	99.1	98.6
Cytoclone	75.5	97.8	88.9	94.4	90.5
Premier	65.4	99.6	97.3	92.3	92.6
Vidas	65.4	100	100	92.6	89.5

[a] In case of indeterminate results, specimens (stool aliquots stored at −80°C) were retested. The second result was considered definitive and was used to calculate statistical parameters.

extra time for isolation of the bacterium; although this method gave excellent results, it is not suitable for clinical applications. However, Gerding et al. (12) have reported that 11% of toxigenic-culture-positive, cytotoxicity test-negative stools were associated with pseudomembranes on lower gastrointestinal endoscopy. The difference between the sensitivities of the cytotoxicity test and toxigenic culture can be explained by the sampling problems inherent in a nonhomogeneous body fluid such as stool (27). In our study, 38 toxigenic *C. difficile* strains were identified; four of these (7.7% of CDAD cases) were from patients who met the case definition for CDAD but were negative by the other tests. Similar results were recently reported by De Girolami et al. (8), who found that 9% of patients harboring toxigenic *C. difficile* had a negative immunoassay result despite a clinical course consistent with CDAD. Two toxigenic *C. difficile* strains were isolated from patients whose clinical courses were not consistent with CDAD; these strains were considered to be transiently colonizing the gastrointestinal tract.

We also identified 18 patients (6.3%) harboring nontoxigenic *C. difficile* strains. This incidence is high compared with those given in previous reports (29, 30) and may be explained by the use of a selective medium containing sodium taurocholate, which enhances spore recovery. High rates of asymptomatic carriage can be observed among hospitalized patients who acquire strains by nosocomial transmission (14, 22, 24).

EIA methods were first used for enterotoxin and cytotoxin detection some time ago, but commercial kits became available only recently. In 1988, Premier was the first EIA based on monoclonal antibodies against toxin A to be approved by the Food and Drug Administration. These EIAs, which are simpler than the cytotoxicity assay and toxigenic culture, are also gaining in popularity because of their rapidity (<3 h). There are small technical differences between the three tests. Vidas is automated, whereas Premier and Cytoclone require manual washes, addition of reagents, aspiration of microwells, etc. Diluted stools are directly analyzed in Premier, whereas Vidas and Cytoclone require initial centrifugation or filtration steps.

TABLE 4. Comparison of EIAs and cytotoxicity assay

Test	Sensitivity (%)	Specificity (%)	Predictive value		Uninterpretable (%)
			Positive (%)	Negative (%)	
Cytoclone	79.6	97.3	86.7	95.7	3.2
Premier	72.5	99.5	97.4	94.3	0.3
Vidas	68	100	100	93.4	2.5

In this study, the sensitivity of the EIAs ranged from 65.4% (Vidas and Premier) to 75.5% (Cytoclone). These results are consistent with the sensitivity of 84.5% for Cytoclone and 69% for Premier found in a study using the same criteria for the diagnosis of CDAD (10). The sensitivity of Vidas has been evaluated as 52% by Shanholtzer et al. (27), but a high percentage of tests (19%) gave uninterpretable results. Estimates of the sensitivity of Premier range from 69 to 89.5% (2, 8–10). The higher sensitivity of Cytoclone versus Premier and Vidas may be explained by its ability to detect both toxin A and toxin B rather than just toxin A. Indeed, toxigenic strains of *C. difficile* produce toxins A and B in roughly equivalent amounts (17). Another explanation involves the use of the streptavidin-biotin system, which can enhance the antigen-antibody detection signal.

Uninterpretable results were more frequent in Cytoclone (6.7%) and Premier (5.3%) than in Vidas (3.8%) in the initial tests. In Premier, all but one of the indeterminate results were resolved by retesting, whereas with Cytoclone and Vidas, most indeterminate results remained indeterminate even after retesting.

All the EIAs had excellent specificity: 99.6% for Premier, 97.8% for Cytoclone, and 100% for Vidas. The good negative and positive predictive values of the EIAs make these tests particularly suitable for rapid screening of stool specimens and whenever same-day reporting is deemed essential.

In conclusion, the three EIAs are rapid, easy to use, and suitable for batch runs. However, because of their lack of sensitivity and the high rate of indeterminate results, EIAs cannot be used as the sole laboratory method for detecting *C. difficile* toxins; results often require confirmation by the stool cytotoxicity assay. Cytotoxicity test and toxigenic culture still remain the most sensitive means for diagnosing CDAD, although they take more time to perform and lack standardization.

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