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Laboratory Diagnosis of *Clostridium difficile*-Associated Gastrointestinal Disease: Comparison of a Monoclonal Antibody Enzyme Immunoassay for Toxins A and B with a Monoclonal Antibody Enzyme Immunoassay for Toxin A Only and Two Cytotoxicity Assays

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A total of 320 stool specimens obtained from 262 patients suspected of having *Clostridium difficile*-associated gastrointestinal disease were examined with two cytotoxicity assays (CTAs) and two commercially available enzyme immunoassays (EIAs). The CTAs were an in-house-developed procedure (University of Massachusetts Medical Center [UMMC], Worcester, Mass.) and a commercial test (Bartels CTA; Baxter Healthcare Corp., West Sacramento, Calif.). One EIA was a monoclonal antibody-based assay for *C. difficile* toxins A and B (Cambridge Biotech Corp. [CBC], Worcester, Mass.). The other EIA employed monoclonal antibodies directed against only toxin A (Meridian Diagnostics, Cincinnati, Ohio). True-positive and true-negative results were defined on the basis of the results of the four assays, clinical assessments of patients, and the results of other laboratory tests. The sensitivities of the four assays were as follows: Bartels CTA, 100%; UMMC CTA, 97.2%; CBC EIA, 84.5%; and Meridian EIA, 69.0%. The Bartels CTA demonstrated a specificity of 99.2%. The other three assays had a specificity of 100%.

A variety of procedures have been employed for the laboratory diagnosis of *Clostridium difficile*-associated gastrointestinal disease (CAD). These procedures include a latex agglutination test for a clostridial surface enzyme, glutamate dehydrogenase (17, 29), enzyme immunoassays (EIAs) for toxins A and B (3, 15, 19, 20), culture methods for *C. difficile* (10, 30, 36), and cell culture cytotoxicity assays (CTAs) (5, 30, 36, 38). In general, CTA has been regarded as the best single laboratory test for CAD.

Recently, a commercial EIA in which both toxins A and B are detected by using monoclonal antibodies was approved by the Food and Drug Administration (FDA). The intent of the current study was to compare this EIA (Cambridge Biotech Corp. [CBC], Worcester, Mass.) with another commercially available monoclonal antibody EIA in which only toxin A is detected (Meridian Diagnostics, Cincinnati, Ohio). In addition, all specimens were also tested with two microdilution CTAs. One of these is an FDA-approved commercially available CTA (Bartels CTA; Baxter Healthcare Corp., West Sacramento, Calif.); the other was developed in-house (University of Massachusetts Medical Center [UMMC], Worcester, Mass.).

The results of initial tests with the four assays (in selected cases, duplicate testing and testing of second stool specimens), ancillary laboratory procedures, and clinical evaluations of study patients were all used to define true-positive and true-negative stool samples. In this way, results obtained with each of the four toxin assays could be evaluated in light of a presumed reference standard as a means of defining true sensitivity, specificity, and positive and negative predictive values.

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MATERIALS AND METHODS

Specimens. Three hundred twenty unselected stool specimens obtained from 262 patients suspected of having CAD were examined over a 3-month period in 1991. Rectal swab specimens were excluded. Two hundred fifteen specimens were obtained from patients hospitalized at the UMMC. Of the remaining 105 specimens, 23 were obtained from UMMC outpatients and 82 were referred specimens submitted by community hospitals in central Massachusetts. All specimens were maintained at 4°C upon receipt in the laboratory and processed within 24 h of collection. At the time of processing, an aliquot of each specimen was frozen at −70°C. In selected instances, repeat testing was performed on this aliquot after it had been thawed to ca. 25°C.

Assays. The Bartels CTA was performed according to the manufacturer's instructions. Briefly, specimens were initially emulsified in sample buffer and centrifuged at 5,000 × *g* for 10 min, and then the supernatant was passed through a 0.45-μm-pore-size filter. The filtrate was tested by using human foreskin fibroblast cells in the wells of a microtiter tray at a final specimen dilution of 1:40. Cells were incubated at 35°C and examined at 100× magnification after 24 and 48 h for any characteristic cytopathic effect which was neutralized by a polyclonal *C. difficile* toxin antibody provided by the manufacturer.

The UMMC CTA was also performed in a microtiter format by using human diploid fibroblasts (MRC-5 cells). Specimens were initially emulsified in phosphate-buffered saline and then centrifuged at 5,000 × *g* at 4°C for 10 min. The supernatant was mixed with equal parts of an antibiotic solution containing amphotericin B (final concentration, 2.5

μg/ml), gentamicin (100 μg/ml), streptomycin (500 μg/ml), and penicillin (500 μg/ml) and incubated at room temperature for 30 min. An aliquot of this suspension was added to the wells of the microtiter tray and incubated at 35°C. The final dilution of specimen varied between 1:16 and 1:32. Cells were examined at 100× magnification after 24 and 48 h for any characteristic cytopathic effect that was neutralized by polyclonal goat anti-*C. difficile* toxins A and B (Tech Labs, Inc., Blacksburg, Va.).

The Meridian EIA was performed according to the instructions of the manufacturer. The specimen, tested at a final dilution of 1:10, was emulsified in buffer provided by the manufacturer and then added to the wells of a microtiter tray coated with polyclonal antibody reactive with *C. difficile* toxin A. The detecting antibody was horseradish peroxidase-labelled monoclonal antibody also reactive with toxin A. Optical density readings were determined with a spectrophotometer at a wavelength of 450 nm. The optical density values were used to define results as follows: <0.100, negative; 0.100 to 0.149, indeterminant; and ≥0.150, positive. The Meridian EIA required ca. 135 min to perform.

The CBC EIA was also performed according to the manufacturer's instructions. The specimen was initially processed by centrifugation and then tested at a final dilution of 1:5. The capture antibodies in the CBC EIA were monoclonal antibodies reactive with both toxin A and toxin B. The detecting antibodies were biotinylated polyclonal goat anti-toxin A and anti-toxin B developed using a streptavidin-horseradish peroxidase conjugate. Optical density readings were determined spectrophotometrically at a wavelength of 450 nm. Interpretation of results was as follows: <0.200, negative; 0.200 to 0.249, indeterminant; and ≥0.250, positive. The CBC EIA required ca. 180 min to perform.

Clinical assessment. Selected patients were evaluated for the likelihood of having CAD on the basis of a slightly modified version of the criteria of Peterson et al. (30). To be considered likely to have CAD, patients needed to have diarrhea (defined as three or more loose or watery stools a day) for at least 2 days (24), to have received antimicrobial therapy within a period of 6 weeks prior to the onset of diarrhea, to have had no other recognized enteric pathogens, and to have demonstrated apparent improvement in symptoms in response to discontinuation of the putative offending antimicrobial agent and/or institution of therapy with metronidazole or vancomycin. Three patients also underwent colonoscopy with observational findings consistent with CAD.

Statistical analysis. Calculations of sensitivity, specificity, and positive and negative predictive values were accomplished as described by Galen and Gambino (9).

RESULTS

A total of 320 stool specimens obtained from 262 patients were examined in this study. Among these, 244 specimens yielded negative results in all four *C. difficile* toxin assays and were considered true negatives. Forty-eight specimens were positive by all four assays and were considered truly positive for *C. difficile*. In the remaining 28 specimens, discordant results were obtained with at least one assay (Table 1). In these 28 cases, the initial specimen was repeat tested by all four assays, a second specimen was obtained from the patient and tested by all four procedures, and a clinical assessment of the patient was made with the aim of defining the likelihood of CAD. The information derived from this supplemental testing and clinical evaluation indicated that 23 of these 28 specimens were in fact positive for *C. difficile* toxin; the other 5 were defined as negative (Table 1). Collectively then, this study consisted of 249 negative and 71 positive stool samples, from 202 and 60 different patients, respectively.

TABLE 1. Results obtained with specimens in which there was disagreement among the four *C. difficile* toxin assays

CAD status and no. of patients	Result[a] obtained with:			
	Bartels CTA	UMMC CTA	CBC EIA	Meridian EIA
Positive				
1	+	+	+	I(+)
1	+	+	+	I(−)
4	+	+	+	−(+)
3	+	+	+	−(+)
1	+	+	+(−)	−
1	+	+	I(+)	−(+)
1	+	+	I(+)	−
1	+	+	I(−)	I(−)
1	+	+	−(+)	−(+)
1	+	+	−(I)	−
6	+	+	−	−
1	+	−(+)	−	−
1	+	−	−	−
Negative				
1	+(−)	−	−	−
1	+	−	−	−
1	−	−	I(−)	I(−)
1	−	−	I(−)	−
1	−	−	−	I(−)

[a] +, positive; −, negative; I, indeterminant. The results in parentheses are the results of repeat tests performed on initial specimens when the results of such secondary testing were different from the results obtained with the first test. When the results of the first and second tests were the same, only the initial result is listed.

The results obtained with all four *C. difficile* toxin assays are listed in Table 2. The Bartels CTA detected all 71 positive stools but gave false-positive results in two cases. Conversely, the UMMC CTA yielded no false-positive results but gave false-negative results in two cases. The CBC EIA gave negative and indeterminant results with 10 and 3 of the 71 *C. difficile*-positive stools, respectively. Among the 249 *C. difficile*-negative specimens, 247 were negative and 2 were indeterminant by the CBC EIA. Twenty false-negative

TABLE 2. Results obtained with four *C. difficile* toxin assays

CAD status (no. of patients)	No. of specimens yielding indicated result[a] upon initial testing with:									
	Bartels CTA		UMMC CTA		CBC EIA			Meridian EIA		
	+	−	+	−	+	−	I	+	−	I
Positive (71)	71	0	69	2	58	10	3[b]	48	20	3[d]
Negative (249)	2	247	0	249	0	247	2[c]	0	247	2[e]

[a] +, positive; −, negative; I, indeterminant.
[b] Repeat testing on two of these specimens with the CBC EIA yielded a positive result; repeat testing on the third specimen was negative.
[c] Repeat testing on these two specimens with the CBC EIA yielded negative results.
[d] One of these specimens was positive with the Meridian EIA upon repeat testing, the other two were negative.
[e] Repeat testing on these two specimens with the Meridian EIA yielded negative results.

TABLE 3. Comparison of statistical parameters for four *C. difficile* toxin assays[a]

Assay	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Bartels CTA	100	99.2	97.2	100
UMMC CTA	97.2	100	100	99.2
CBC EIA	84.5	100	100	95.8
Meridian EIA	69.0	100	100	91.9

[a] When an indeterminant result was obtained upon initial testing, the results of repeat tests on that specimen were used to calculate statistical parameters.

and three indeterminant results were obtained with the Meridian EIA with *C. difficile*-positive stool specimens. As was the case with the CBC EIA, 247 negative and 2 indeterminant results were obtained with the Meridian EIA from the 249 *C. difficile*-negative stools. Calculations of sensitivity, specificity, and negative and positive predictive values with the four assays are listed in Table 3. For the purposes of these calculations, the results of repeat tests on stools initially yielding indeterminant results were considered definitive.

Another approach to assessing the relative sensitivities of the four *C. difficile* toxin assays examined in this study was to compare the performance of these assays with the 23 stool specimens that were judged to be positive for *C. difficile* but which had yielded at least one discrepant result (Table 1). All 23 of these specimens were positive in the Bartels CTA, 21 of 23 were positive with the UMMC CTA, 10 were positive and 3 were indeterminant with the CBC EIA, and none were positive with the Meridian EIA (3 indeterminant results). Furthermore, among these 23 specimens, there were no cases when the Meridian EIA was positive when the CBC EIA was negative, the CBC EIA was positive when the UMMC CTA was negative, or the UMMC test was positive when the Bartels CTA was negative.

In addition, there were two subsets of patients among this group of 23 in which the course of CAD and the results of testing additional stool specimens was very illustrative. One group of eight patients (group A [Table 4]) had previously been diagnosed as having CAD, had had their offending antimicrobial agent(s) discontinued, and had been started on specific therapy (metronidazole, five patients; vancomycin, three patients) at the time the stool specimen was obtained that yielded discordant results. The symptoms of all eight patients had improved by the time the second stool specimens were obtained. The results of testing on initial and second stool specimens in this group of eight patients are depicted in Table 4. Clearly with the initial specimen, there was a tendency for both CTAs to be positive, the CBC assay to be positive or indeterminant, and the Meridian assay to be falsely negative. All four assays generally converted to negative when second specimens were tested.

The second subset (group B [Table 4]) consisted of seven patients who appeared to have had stool specimens collected very early in the course of their disease. In all seven cases, second specimens were provided prior to administration of specific therapy. Four of these patients were still receiving the putative offending antimicrobial agent(s) at the time the second sample was obtained, while three had had them discontinued. The symptoms of all seven patients were unchanged or had worsened by the time the second specimen was collected. *C. difficile* toxin assays on initial specimens from these seven patients tended to give positive results in the two CTAs but false-negative results in the two EIAs (Table 4); there were, however, two positive CBC EIA results in this group. Testing of second specimens from these patients generally yielded positive results in all four assays, although the Meridian EIA was negative in two cases.

DISCUSSION

Definitive laboratory diagnosis of CAD is problematic. Culture recovery of *C. difficile* from fecal specimens is of questionable significance even when specimens have been obtained from patients with a compatible illness, both epidemiologically and clinically, and when isolates are shown to produce toxin. This is because toxigenic strains of *C. difficile* are well documented to at least transiently colonize the gastrointestinal tract of asymptomatic individuals (4, 5, 13,

TABLE 4. Results obtained with selected first and second specimens when testing of initial specimens yielded discordant results

Patient group[a] and no. of patients	Result[b] of tests on:							
	Initial specimen				Second specimen			
	Bartels CTA	UMMC CTA	CBC EIA	Meridian EIA	Bartels CTA	UMMC CTA	CBC EIA	Meridian EIA
A								
1	+	+	+	I(+)	+	−	−	−
2	+	+	+	−(+)	−	−	−	−
2	+	+	+	−	−	−	−	−
1	+	+	I(+)	−(+)	+	+	−	−
1	+	+	I(+)	−	−	−	−	−
1	+	+	−(+)	−(+)	−	−	−	−
B								
1	+	+	+	−(+)	+	+	+	−
1	+	+	+	−	+	+	+	+
1	+	+	I(−)	I(−)	+	+	+	+
3	+	+	−	−	+	+	+	+
1	+	−(+)	−	−	+	+	+	−

[a] Patient groups are described in Results.

[b] +, positive; −, negative; I, indeterminant. The results in parentheses are the results of repeat tests performed on initial specimens when the results of such secondary testing were different from the results obtained with the first test. When the results of the first and second tests were the same, only the initial test result is listed.

18, 26). High rates of asymptomatic carriage have been described in neonates (2, 7, 31), patients with cystic fibrosis (28, 37), and hospitalized patients who acquire their strains via nosocomial transmission (14, 22, 24, 26, 35).

Direct detection of *C. difficile* toxin B, a potent cytotoxin, by cell culture assay is highly predictive in patients with characteristic epidemiology and clinical manifestations. The results of toxin B CTA, however, are dependent at least to some extent upon the cell line employed (23), the age of cells (33), the manner in which the stool specimen was processed initially and the test format (36). The sensitivity of CTA has been reported to vary between 67 and 78%; the specificity varies between 95 and 99% (12, 29, 30, 36). Additional limitations of toxin B CTA include the problem of interfering substances present in stool specimens, the prolonged time period required to obtain a test result (24 to 48 h), expense, and the technical complexities associated with any cell culture assay. CTA, however, remains the best proven single method for establishing a laboratory diagnosis of *C. difficile* disease. One microtiter CTA (Bartels) is FDA approved and commercially available (27, 38).

Alternative approaches to the diagnosis of CAD which have been developed recently include a commercially available latex agglutination test (29) and EIAs for direct application to stool samples (3, 15, 19, 20). The first latex agglutination test, the Culturette brand CDT, was introduced in 1985 by Marion Scientific, Inc. (Kansas City, Kans.) and is now manufactured and distributed by Becton-Dickinson Microbiology Systems, Inc. (Cockeysville, Md.). This procedure, originally marketed as a test for *C. difficile* toxin A, was subsequently shown to detect a *C. difficile* protein antigen that was common to several anaerobic bacteria (16, 21, 25). It has recently been suggested that the target antigen of the Becton-Dickinson *C. difficile* latex agglutination test is a clostridial surface enzyme, glutamate dehydrogenase (17). Not surprisingly, the latex test is relatively nonspecific (89 to 97%); estimates of its sensitivity have ranged from 68 to 92% (6, 12, 29, 30, 32).

In 1988, the first enzyme immunoassay for *C. difficile* toxin became available commercially (Meridian Diagnostics). This is a monoclonal antibody-based assay for *C. difficile* toxin A. Estimates of the sensitivity of the Meridian EIA have ranged from 40 to 92%, whereas estimates of its specificity have ranged from 96 to 99.5% (1, 11, 34). Three additional commercial EIAs that detect toxin A and employ monoclonal antibodies are either currently available or are pending FDA approval. These assays have not been extensively studied. The CBC EIA examined in this study is the only commercially available test for both toxins A and B.

The intent of the current investigation was to compare four *C. difficile* toxin assays as means for establishing a diagnosis of CAD. These included two CTAs and two EIAs. Criteria were established that were used to define true-positive and true-negative samples. These criteria included the results of laboratory studies as well as clinical evaluations. In this manner, each of the assays could be assessed relative to a "gold standard," rather than merely in comparison to the results of other assays.

All of the assays had excellent specificities. Only two false-positive results were obtained, both of these with the Bartels CTA. Although the reproducibility of the assays was not evaluated, the sensitivity varied: Bartels CTA, 100%; UMMC CTA, 97.2%; CBC EIA, 84.5%, and Meridian EIA, 69.0%. The enhanced sensitivity of the CBC EIA versus the Meridian assay may be explained by the lower final specimen dilution used in the CBC EIA (1:5) than that in the Meridian EIA (1:10). Another possible explanation is the ability of the CBC EIA to detect *C. difficile* toxins A and B, rather than merely toxin A. There is good evidence that strains of *C. difficile* are either completely nontoxigenic or produce both toxins A and B (8, 18). If it is assumed that both toxins are produced in roughly equivalent amounts by toxigenic strains, then an EIA which detects both toxins A and B would be expected to be more sensitive than an EIA for toxin A only. This hypothesis is, of course, predicated on the assumption that the limit sensitivity of each assay (as determined by the smallest amount of toxin detectable) is comparable.

It should also be pointed out that nine specimens yielding false-negative results on initial testing with the Meridian EIA tested positive with this assay on repeat testing. The only salient difference between initial and repeat testing was that the second test was performed on stool that had been frozen at −70°C and thawed once. It is possible that freezing and thawing actually enhances the reactivity of the Meridian EIA.

In conclusion, the results of this study indicate that CTA is, in general, a more sensitive means for establishing a laboratory diagnosis of CAD than EIA. Of the two EIAs examined in this study, the CBC EIA was more sensitive than the Meridian EIA. Both assays yielded no false-positive results and thus, were 100% specific. Furthermore, the differences in sensitivities between the CTAs and the CBC EIA were relatively small. Although in this study, the CBC EIA missed 14% of the true-positive specimens, this assay merits consideration as a primary test for CAD, particularly in laboratories that lack the facilities and/or expertise to perform CTA.

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