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Comparison of a Dot Immunobinding Assay, Latex Agglutination, and Cytotoxin Assay for Laboratory Diagnosis of *Clostridium difficile*-Associated Diarrhea

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C. *diff*-CUBE, a dot immunobinding assay (DIA) (Difco Laboratories, Ann Arbor, Mich.) for detection of *Clostridium difficile* toxin A in stool specimens, was compared with latex agglutination (LA) (Marion Laboratories, Kansas City, Mo.) and cytotoxin assay (CTA) for the laboratory diagnosis of *C. difficile*-associated diarrhea. A total of 200 stool specimens collected from 169 patients with suspected *C. difficile* diarrhea were tested. Of the 198 specimens evaluated by all three methods, 36 (18%) from 36 patients were positive by one or more of the tests. Twenty-five, 26, and 23 specimens were positive by CTA, DIA, and LA, respectively; 14 were positive by all three methods. Eight specimens yielded nonspecific LA test results; all eight were negative by CTA, and one was positive by DIA. DIA results agreed with CTA results in 183 (92%) cases and with LA results in 175 (88%) cases. CTA and LA results agreed in 179 (90%) cases. Freezing of the specimen did not appear to adversely affect either the DIA or LA test. These preliminary results suggest that *C. diff*-CUBE may be useful as a rapid screen for the diagnosis of *C. difficile*-associated diarrhea. However, for optimum laboratory diagnosis, further testing of all stools that are negative by DIA is warranted.

Clostridium difficile is the most common cause of pseudomembranous colitis and is responsible for approximately one-fifth of all cases of antibiotic-associated diarrhea. The reference method for laboratory diagnosis of *C. difficile*-associated diarrhea has been detection of toxin B by cytotoxin (tissue culture) assay (2). Since many clinical microbiology laboratories do not perform tissue cell culture, alternative diagnostic tests have been evaluated. Counterimmunoelectrophoresis, enzyme-linked immunosorbent assay, gas-liquid chromatography, and latex agglutination have been used in a research setting (1, 5, 6, 8, 9, 12). In 1986, Marion Laboratories, Inc. (Kansas City, Mo.) marketed a latex agglutination kit (Culturette Brand CDT *C. difficile* test) specific for *C. difficile* toxin A. The test has since been proven not specific for toxin A (10); however, in clinical studies, this latex test has correlated well with the presence of *C. difficile*-associated diarrhea (7, 14). Difco Laboratories (Ann Arbor, Mich.) recently developed a dot immunobinding assay (*C. diff*-CUBE) for rapid detection of *C. difficile* toxin A in stool specimens. This report describes an evaluation of the dot immunobinding assay as a laboratory test for the diagnosis of *C. difficile*-associated diarrhea. Results were compared with those of the Marion latex agglutination test and the cytotoxin assay with McCoy cells.

MATERIALS AND METHODS

Specimens. A total of 200 stool specimens collected from 169 patients in whom the diagnosis of *C. difficile*-associated diarrhea was suspected were tested. Forty-four of the specimens were received frozen through the regional laboratory; these were stored at −70°C prior to testing. All other specimens were held at 4°C and were tested within 48 h of collection.

Cytotoxin Assay. Ninety-six-well tissue culture plates seeded with McCoy cells (American Type Culture Collection CCL2, Rockville, Md.) were inoculated with stool filtrates. Formed and soft stool samples were mixed with an equal volume of *C. difficile* Toxi-titer diluent (Bartles Immunodiagnostic Supply, Bellevue, Calif.). Liquid stools were not diluted. Fluid stool material was centrifuged at 2,500 × *g* for 20 min, and the supernatant was passed through a sterile, disposable 0.45-μm-pore-size filter (Nalgene Co., Rochester, N.Y.). The filtrate was frozen at −20°C until tested.

At the time of testing, a mixture of stool filtrate (0.05 ml) and growth medium (0.15 ml) was serially diluted. A 0.1-ml volume of *C. difficile* antitoxin (VPI Anaerobe Laboratories, Blacksburg, Va.), diluted 1:25 in phosphate-buffered saline, was added to the 1:8 dilution of stool filtrate. Then 0.1 ml of each dilution (with and without antitoxin) was added aseptically to the 96-well microdilution plate containing McCoy cells. A toxin control, antitoxin control, neutralization control, and cell culture control were included with each specimen run (4). The plate was covered, incubated at 35°C in 7% CO_2 for 24 h, and subsequently examined for cytopathic effect. A titer of greater than 1:8 was considered positive (4).

Dot immunobinding assay. The *C. diff*-CUBE test was performed according to the directions of the manufacturer. Watery stools were prepared by placing an equal volume (approximately 200 μl) of specimen diluent (provided by the manufacturer) in a tube, and solid stools were prepared by placing approximately 0.1 g in a tube containing 0.5 ml of specimen diluent. Specimens were vortexed and centrifuged for 15 min at 2,500 × *g*. With the transfer pipette provided, three drops of the specimen supernatant were added to the surface of an individual membrane cassette with a prefilter in place. After the materials passed through the filter, the prefilter was removed and discarded and one drop of mouse monoclonal antibody was added to the membrane surface. This was allowed to react for 1 min, after which time two drops of anti-mouse immunoglobulin G horseradish peroxidase concentrate were added to the membrane surface. After incubation for 1 min, 10 drops of wash solution were added

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TABLE 1. Results of *C. difficile* cytotoxin assay, dot immunobinding assay, and latex agglutination

Test result by[a]:			No. of specimens
CTA	DIA	LA	
−	−	−	155
+	+	+	14
−	−	+	3
−	+	−	6
+	−	+	4
+	+	−	4
−	+	+	2
+	−	−	2
−	−	NSPA	7
−	+	NSPA	1
NSPT	−	−	1
ND[b]	−	−	1

[a] Abbreviations: CTA, *C. difficile* cytotoxin assay; DIA, dot immunobinding assay; LA, latex agglutination; NSPA, nonspecific agglutination; ND, not done; NSPT, nonspecific toxicity.

[b] Recovery of a small quantity of supernatant that would not pass through the filter precluded performance of the CTA.

to the membrane. When all of the materials had passed through the membrane, three drops of chromogen-substrate reagent were added. A homogeneous blue or blue-green dot appearing in the center of the membrane surface within a 3-min observation period was considered positive for *C. difficile* toxin A.

Latex agglutination. The latex agglutination test was performed and interpreted according to the directions of the manufacturer (14). Stool and test buffer was mixed and centrifuged. One drop of stool supernatant was placed in both the "test" and "negative" circles of the agglutination slide, and detection reagent and negative control reagent, respectively, were added and mixed. To the positive control circle, the positive control and detection reagent were added and mixed. The entire slide was rotated and subsequently examined for agglutination. Interpretation was as follows: positive, stronger agglutination in the test than in the negative circle; negative, no agglutination in either circle; and nonspecific agglutination, equal agglutination in both circles. All specimens initially yielding nonspecific agglutination were diluted 1:2 and retested. The final interpretation was based on the above guidelines.

RESULTS

Results of the cytotoxin assay, dot immunobinding assay, and latex agglutination tests are shown in Table 1. Of the 200 specimens, 36 (18%) were positive by at least one method. A total of 24 (12.0%) were positive by cytotoxin assay, 27 (13.5%) were positive by dot immunobinding assay, and 23 (12.0%) were positive by latex agglutination. Of the 36 positive specimens, 14 (38.9%) were positive by all three methods. Dot immunobinding assay results agreed with cytotoxin assay results for 183 (92.0%) specimens and with latex agglutination results for 175 (87.5%) specimens. Cytotoxin assay and latex agglutination results agreed for 179 (89.5%) specimens. The latex agglutination test yielded nonspecific results for eight specimens. All eight were negative by cytotoxin assay, and one was positive by the dot immunoblotting assay.

A total of 44 specimens were frozen prior to testing. Twelve (27.3%) of these were positive by at least one method. Six (50.0%) of the 12 were positive by all three tests. Cytotoxin assay results agreed with dot immunobinding assay results for 40 (90.9%) specimens and with latex agglutination results for 42 (95.4%) specimens. Two specimens each were positive by dot immunobinding assay and latex agglutination alone.

DISCUSSION

C. difficile-associated diarrhea is a common nosocomial infection. Because the carrier rate for *C. difficile* in hospitalized patients approaches 20% (13), the cytotoxin assay rather than culture has been considered to be the best method for diagnosing *C. difficile* disease (2). Limitations of the cytotoxin assay include the requirement for tissue culture facilities and a 24-h turn-around time. Moreover, data from more recent studies have suggested that anaerobic culture of stool samples specifically for *C. difficile* is more sensitive than is cytotoxin assay for the diagnosis of *C. difficile*-associated diarrhea (7, 11). Given these limitations, a reliable, rapid, inexpensive, and technically simple diagnostic test is desirable. The Marion latex agglutination test, although not specific for toxin A as initially suggested, is rapid, easy to perform, and appears to correlate with *C. difficile*-associated disease (7, 14). The sensitivity of the latex test, however, has ranged from 68 to 90% (3, 7, 11).

The primary objective of this study was to evaluate a recently developed rapid test—a dot immunobinding assay—as a laboratory tool with which to diagnose *C. difficile*-associated diarrhea. Dot immunobinding assay results were compared to those of cytotoxin assay and latex agglutination. Because the dot immunobinding assay was developed to detect *C. difficile* toxin A and not the presence of the organism, bacterial culture was not included in the evaluation. This study design, however, is limited by the fact that neither the cytotoxin assay nor latex agglutination will detect all cases of *C. difficile*-associated disease. For optimal identification of patients with disease, clinical data should be prospectively collected. Since that was not done in this study, an attempt was made to determine the presence or absence of disease when a discrepancy occurred among test results by retrospectively reviewing in-patient charts. Patients were considered to have *C. difficile*-associated disease if they had diarrhea with stool specimens negative for enteric pathogens, recent antibiotic use, or endoscopy-proven colitis (8) or all three. This approach, however, was limited by the inaccessibility of records of patients from other hospitals and the case definition itself, since a *C. difficile* carrier with diarrhea due to other drugs or tube feedings might falsely be considered to have disease, and a patient with chemotherapy-induced disease would be excluded.

Overall, agreement among the three tests was good. The dot immunobinding assay agreed with the cytotoxin assay results in 92% of the total cases and with latex agglutination results in 88% of all cases. Latex agglutination and cytotoxin assay results agreed for 90% of specimens.

There were advantages and disadvantages associated with each of the rapid tests evaluated. The latex test requires fewer reagents. However, occasional specimens (4% in our evaluation) yielded nonspecific agglutination and therefore required further evaluation by cytotoxin assay or some other method. The frequency of nonspecific agglutination found by other institutions is unknown, since this problem was not specifically mentioned by others evaluating the test (2, 7, 11). With the dot immunobinding assay, on the other hand, results were either positive or negative, which offers a distinct advantage. Dense staining of the cassette membrane

by an especially dark supernatant or the presence of an excessive amount of debris could cause difficulty in interpretation of the dot immunobinding test result; however, this was a significant problem with only two (1%) specimens.

In this report, the preliminary data suggest that the dot immunobinding assay can be used as a laboratory screening test for presumptive diagnosis of *C. difficile*-associated disease. A more extensive evaluation including culture and prospectively collected clinical information is warranted.

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