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Multicenter Evaluation of Four Methods for *Clostridium difficile* Detection: ImmunoCard *C. difficile*, Cytotoxin Assay, Culture, and Latex Agglutination

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A three-center study was undertaken to compare several test methods for the detection of *Clostridium difficile*, associated toxin, or related markers by using 927 stool specimens. Methods included direct assay of cytotoxin in stool by tissue culture, *C. difficile* bacterial culture followed by cytotoxin assay, bacterial culture alone, latex agglutination assay, and the ImmunoCard *C. difficile* test (Meridian Diagnostics, Inc.). The sensitivities, as determined against direct cytotoxin assay results, of the ImmunoCard *C. difficile* and latex agglutination assays were 84 and 67%, respectively (92 and 77%, respectively, when adjusted for bacterial culture outcomes). Evaluation for *C. difficile*-associated disease (CDAD) among 864 patients was based on clinical criteria for antibiotic-associated diarrhea combined with laboratory evidence of toxin or toxin-producing *C. difficile* in stool specimens. The sensitivity of each test method for screening of CDAD was as follows: bacterial culture, 95%; culture with cytotoxin assay of isolates, 90%; ImmunoCard *C. difficile* test, 83%; cytotoxin assay 82%; and latex agglutination assay, 67% ($P \leq 0.05$ versus all other methods). The standard deviations of the test sensitivity statistics between study sites were ranked as follows: cytotoxin assay (±3.1%) < ImmunoCard *C. difficile* test (±5.7%) < latex agglutination assay (±12.3%) < culture (±24.7%) < culture with cytotoxin assay (±28.0%). The data support the use of the ImmunoCard *C. difficile* test as an adjunct for the diagnosis of CDAD.

Since the discovery of the causative role of *Clostridium difficile* toxin in antibiotic-associated diarrhea (AAD) and pseudomembranous colitis (2, 3, 10, 15, 21), a variety of laboratory methods have been developed to detect the presence of the organism or its related toxins (toxins A and B). The spectrum of available analytical techniques can be grouped into two main categories, either toxin dependent or toxin independent. Toxin-dependent methods include tissue culture assay for the presence of cytotoxin, culture for the organism with follow-up toxin testing (toxigenic culture), and a variety of enzyme immunoassays (EIAs) directed toward the detection of toxin A or toxin B, or both. Such methods exploit the role of the toxin(s) in the causation of disease, a role well established in both animal models (6, 22) and human studies (8, 10, 13, 14, 16). Toxin-independent methods for the detection of *C. difficile* include culture of stool specimens under anaerobic conditions, latex agglutination, and a new ImmunoCard *C. difficile* test (Meridian Diagnostics, Inc., Cincinnati, Ohio). While culture depends on the presence of viable vegetative cells or spores in the stool, the latex agglutination assay and ImmunoCard *C. difficile* test methods detect a marker antigen for *C. difficile*. This antigen has been identified as the enzyme glutamate dehydrogenase (GDH) (20, 27) and is present in all *C. difficile* isolates, regardless of their toxin-producing ability.

The current study was undertaken to evaluate the new ImmunoCard *C. difficile* test and to compare its performance with those of several other methods (culture, culture with toxin assay, cytotoxin assay, and latex agglutination assay) for the detection of *C. difficile*-associated disease (CDAD). In addition, intersite variation was evaluated.

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MATERIALS AND METHODS

Study sites and stool specimens. Nine hundred twenty-seven stool specimens were assayed at three hospital clinical microbiology laboratory study sites. The numbers of specimens (original stool specimens without transport medium) tested at each site were 302 at the University of Cincinnati (UC), 312 at the University of North Carolina (UNC), and 313 at Indiana University at Purdue University of Indiana (IUPUI). All specimens were stored at 4°C for ≤72 h until they were tested.

ImmunoCard *C. difficile* test. The ImmunoCard *C. difficile* test (Meridian Diagnostics, Inc.) method uses antibody directed against *C. difficile* GDH. Testing was performed according to the manufacturer's instructions. Briefly, stool specimens were diluted 1/15 in enzyme conjugate (alkaline phosphatase-conjugated rabbit anti-GDH immunoglobulin G), and 150 μl was added to each of two sample ports. The left lane contained immobilized purified *C. difficile* GDH and served as a sample control. The right lane contained immobilized rabbit anti-GDH immunoglobulin G and served as the sample test lane. The upper reaction ports were then washed (3 drops), substrate (2 drops) was added, and the mixture was allowed to develop for 5 min. The control (left) and test (right) ports were observed for blue color development. Specimen results with no blue color in the control port were interpreted as invalid. Valid results (blue color in control port) were read as either positive (blue) or negative (no color) in the test port.

Latex agglutination assay. Latex agglutination (Culturette CDT *C. difficile*; Becton Dickinson & Co., Cockeysville, Md.) testing was performed according to the manufacturer's instructions.

Cytotoxin assay. At IUPUI and UC, a commercial cytotoxin assay (Bartels Immunodiagnostic Supplies, Inc., Bellevue, Wash.) was used for the present study according to the manufacturer's instructions. At UNC, a previously described method with MRC-5 cells was used (16).

Culture and isotoxin testing. All sites used a commercial selective medium (CCFA plus horse serum; Carr-Scarborough, Stone Mountain, Ga.) for culture. Briefly, swabs were used to sample mixed stool specimens and were streaked onto CCFA plates for isolation. Incubation was carried out anaerobically for 48 h at 35 to 37°C. In general, four to six colonies with characteristics of *C. difficile* (gray colored and spreading with a ground glass appearance) were visualized with a dissecting microscope, subcultured to anaerobic broth (brain heart infusion broth), and incubated for 48 h at 35 to 37°C. Identification of the isolates as

TABLE 1. Comparison of ImmunoCard *C. difficile* and latex agglutination assay results with cytotoxin assay and resolved results by using culture outcomes

Test method	Result	Cytotoxin assay results (no. of specimens)		Performance characteristics (%)				
		Positive	Negative	Sensitivity	Specificity	Correlation	PPV[a]	NPV[b]
ImmunoCard *C. difficile* test	Positive	108 (140)[c]	63 (31)	84 (92)	92 (96)	91 (95)	63 (82)	97 (98)
	Negative	20 (12)	715 (723)					
Latex agglutination assay	Positive	86 (105)	39 (20)	67 (77)	95 (97)	91 (94)	69 (84)	95 (96)
	Negative	42 (31)	734 (745)					

[a] PPV, predictive value of a positive test result.
[b] NPV, predictive value of a negative test result.
[c] Values in parentheses are resolved values by using culture outcomes for discrepant pairs.

C. difficile was confirmed by gas chromatography to show the production of butyric, isobutyric, valeric, isovaleric, and caproic acids. The isolates were tested for in vitro toxin production (isotoxin) by performing a cytotoxin assay on filtered 48-h broths.

Data analysis and patient diagnosis. Patients were defined as having AAD if significant diarrhea (six or more loose stools within 48 h) was noted together with a recent history (within 8 weeks) of treatment with antibiotics or chemotherapeutic agents (8, 18, 25). A patient was categorized as having CDAD if both of the following criteria were fulfilled: (i) a stool specimen yielded a positive result in at least one toxin-dependent *C. difficile* assay and (ii) AAD was present. To determine AAD status, a retrospective chart review was attempted for any patient for whom one or more toxin-dependent tests were found to be positive. In all, chart review was undertaken for 248 (27%) patients. Patients without a toxin-dependent positive test were classified by definition as being CDAD negative. Only specimens from patients for whom a positive or negative CDAD determination could be made were used in test evaluations. Performance statistics were calculated as described by Galen and Gambino (11, 12). Differences between test performance values were calculated as recommended by Ilstrup (17). Latex agglutination assay-indeterminate ($n = 10$) and ImmunoCard *C. difficile* test-invalid ($n = 5$) results were not included in calculations of relative performance.

RESULTS

Of the 927 stool specimens tested, 227 gave positive results in at least one assay. Of these results, 172 (75.8%) were positive by the ImmunoCard *C. difficile* test method, 162 (71.4%) were positive by culture, 129 (56.8%) were positive by cytotoxin assay, 126 (55.5%) were positive by latex agglutination assay, and 123 (54.2%) were positive by toxigenic culture (stools yielding toxin-producing isolates of *C. difficile*).

A common convention when investigating new methods of *C. difficile* detection has been to compare the results with cytotoxin assay results. The performance characteristics of the ImmunoCard *C. difficile* and latex agglutination assays compared with cytotoxin assay results are presented in Table 1. The sensitivity values relative to the cytotoxin assay results were 84 and 67%, respectively ($P \leq 0.05$), while the predictive values of a positive test were 63 and 69%, respectively (the data are not statistically different). Sixty-three specimens positive by the ImmunoCard *C. difficile* test and negative by the cytotoxin assay were reviewed for culture outcomes. Thirty-two of the 63 specimens produced positive culture results, yielding 10 toxigenic and 22 nontoxigenic isolates. Similarly, those 20 specimens which gave ImmunoCard *C. difficile* test-negative, cytotoxin assay-positive results yielded 12 *C. difficile* isolates, all of which produced toxin. Thus, relative to the cytotoxin assay and culture outcomes, the resolved performance of the ImmunoCard *C. difficile* test was 92% sensitive and 95% specific, with a 95% correlation (Table 1). Review of the 39 latex agglutination assay-positive, cytotoxin assay-negative specimens revealed 8 toxigenic and 11 nontoxigenic culture isolates. Latex agglutination assay-negative, cytotoxin assay-positive specimens ($n = 42$) yielded 28 toxigenic and 3 nontoxigenic culture isolates. Resolved performance values for the latex agglutination test relative to cytotoxin assay and culture results were 77% sensitive ($P < 0.05$ versus that of the ImmunoCard *C. difficile* assay) and 97% specific, with a 94% correlation statistic.

Comparison of the results of the test methods with cytotoxin assay results provides a useful benchmark. However, it is the actual diagnostic utility of the test method that is of value. Thus, results of all five test methods were compared with a diagnosis of CDAD (Table 2). A determination of CDAD status was made for 864 of 927 (93.2%) of the specimens. Diagnostic conclusions could not be made for 63 patients whose charts were either incomplete or not available. The prevalence of CDAD was 123 of 864, or 14.2%, consistent with published values (18, 19, 21, 22). The prevalence per site was as follows: IUPUI, 47 of 295 (15.9%); UC, 41 of 261 (15.7%); and UNC, 35 of 308 (11.4%). False-negative and false-positive results were noted by all methods, including the cytotoxin assay and toxigenic culture.

The sensitivities of the ImmunoCard *C. difficile* test for CDAD screening at IUPUI, UC, and UNC were 91, 80, and 78%, respectively, with an overall sensitivity of 83% (standard deviation [SD], 5.7%). The overall sensitivity of the latex agglutination assay was 67% (80, 73, and 51% at IUPUI, UC, and UNC, respectively; SD, 12.3%). The sensitivity of the cytotoxin assay for CDAD was 82% overall (85, 83, and 78% at IUPUI, UC, and UNC, respectively; SD, 3.1%). The sensitivities of culture were 96, 95, and 43% at IUPUI, UC, and UNC, respectively (SD, 24.7%). Statistical analysis revealed that the 43% sensitivity value for culture from one site was significantly different ($P < 0.01$) from the values from the remaining sites. Therefore, the culture results from this site were not used in calculating relative performance values. The overall sensitivity of culture for the detection of CDAD at the two remaining sites was 95%. Similarly, the sensitivities of toxigenic culture for the detection of CDAD at IUPUI, UC, and UNC were 95, 85, and 31%, respectively (SD, 28.0%). The 31% statistical outlier ($P < 0.01$) was not used in calculating the overall sensitivity (90%). The latex agglutination test was significantly less sensitive than any other method for the detection of CDAD ($P < 0.05$). Statistically, the cytotoxin assay and toxigenic culture were both more specific ($P < 0.05$) than either culture or the ImmunoCard *C. difficile* test. Toxigenic culture proved to be the most accurate single method; this was followed by cytotoxin assay, culture, the ImmunoCard *C. difficile* test, and the latex agglutination assay.

DISCUSSION

The present study was undertaken (i) to ascertain the performance and diagnostic value of the new ImmunoCard *C.*

TABLE 2. Comparison of test results with diagnosis of CDAD

Test methods	Test result	No. of specimens with the indicated CDAD result		Performance characteristics (%)[a]				
		Positive	Negative	Sensitivity	Specificity	Correlation	PPV[b]	NPV[c]
ImmunoCard *C. difficile* test	Positive	102	37	83 ± 6.7	95 ± 1.6	93 ± 1.7	73 ± 7.3	97 ± 1.2
	Negative	20	700					
	Invalid[d]	1	4					
Latex agglutination assay	Positive	82	23	67 ± 8.4	97 ± 1.3	93 ± 1.8	78 ± 7.9	95 ± 1.6
	Negative	39	710					
	Indeterminate[e]	2	8					
Cytotoxin assay	Positive	101	8	82 ± 6.8	99 ± 0.8	96 ± 1.2	93 ± 4.9	97 ± 1.2
	Negative	22	733					
Culture[f]	Positive	84	23	95 ± 4.3	95 ± 2.0	95 ± 1.8	78 ± 7.8	99 ± 0.9
	Negative	4	445					
Toxigenic culture[f]	Positive	79	5	90 ± 6.5	99 ± 0.9	97 ± 1.3	94 ± 5.1	98 ± 1.2
	Negative	9	463					

[a] Values are characteristic ±95% confidence interval (18).
[b] PPV, predictive value of a positive test result.
[c] NPV, predictive value of a negative test result.
[d] Invalid test results for the ImmunoCard *C. difficile* test occurred when the left (control) port failed to turn blue.
[e] Indeterminate latex agglutination test results occurred when agglutination occurred in both test and negative wells.
[f] Data from statistical outlier site not included (see text).

difficile test relative to the diagnosis of CDAD, (ii) to permit comparison of different test methodologies with the benchmark cytotoxin assay, and (iii) to evaluate site-to-site variations in performance values. The study design did not incorporate either the latex agglutination test, culture (without isotoxin testing), or the new ImmunoCard *C. difficile* test into any aspect of the diagnosis of CDAD, thus eliminating positive bias for these methods. Conversely, the presence of either toxigenic *C. difficile* or cytotoxin in the stool were bias factors in the case definition (i.e., the presence of toxin or a toxin-producing organism was required for a diagnosis of CDAD).

Culture and culture with isotoxin testing (toxigenic culture) proved to be the most sensitive methods for the diagnosis of CDAD (95 and 90%, respectively). The lower sensitivity of toxigenic culture resulted from four specimens at one site which yielded only nontoxigenic isolates. These four isolates were negative for both cytotoxin and toxin A (Premier *C. difficile* Toxin A EIA; Meridian Diagnostics, Inc.) production. The ImmunoCard *C. difficile* test was shown to have a sensitivity similar to that of the cytotoxin assay (sensitivities, 83 and 82%, respectively), while, as would be expected of a test designed to be a screening test, the predictive value of a positive test was lower for the ImmunoCard *C. difficile* test than for the cytotoxin assay (predictive values of a positive test result, 73 and 93%, respectively). The utility of the ImmunoCard *C. difficile* test would include its use as a simple and rapid screen with good sensitivity for the identification of patients potentially positive for *C. difficile*. Such specimens could be subjected to a definitive, toxin-dependent assay to rule out false-positive screen results. The predictive value of a negative ImmunoCard *C. difficile* test result was equal to that for the cytotoxin assay. The latex agglutination test, however, appeared to be the least sensitive method of those investigated for the detection of CDAD (67%), bringing into question its utility for the diagnosis of CDAD. Although culture alone (without subsequent toxin assay) had a sensitivity of 95% for the diagnosis of CDAD, this approach requires several days for results, is challenging technically (see below), and is therefore unattractive as a screening test.

Evaluating the ImmunoCard *C. difficile* and latex agglutination tests with cytotoxin testing serves as a benchmark for comparison with the results of other studies in which cytotoxin detection rather than the diagnosis of CDAD was used as a reference. The sensitivity of the ImmunoCard *C. difficile* test was 84% relative to the results of the cytotoxin assay, while the latex agglutination test was only 67% sensitive. These results are consistent with recent reports that found the latex agglutination test to be only 60 to 70% sensitive relative to the results of the cytotoxin method (19) and are in contrast to earlier published values of 80 to 90% sensitivity (4, 18, 26). The cause for the apparent drop in sensitivity of the latex agglutination test is not known. Conversely, the ImmunoCard *C. difficile* test's sensitivity (84%) relative to the cytotoxin assay results was fairly high, perhaps reflecting the advantages of an EIA over a latex agglutination technique. The ImmunoCard *C. difficile* test's sensitivity (relative to cytotoxin testing) was similar to published values for microwell toxin A and toxin B EIAs (1, 5, 7–9, 16), although the specificity (92%) was lower because of reaction with the GDH of nontoxigenic strains of *C. difficile.*

The study of site-to site-variation (performance values for comparisons with the diagnosis of CDAD) indicated only slight differences in accuracy and specificity. This observation was due to the high degree of specificity of all the methods evaluated and the large percentage of negative specimens seen in the sample populations. The sensitivity values were the most revealing with regard to intersite variability. The cytotoxin assay had the most consistent sensitivity values (SD, 3.1%); this was followed closely by the ImmunoCard *C. difficile* test (SD, 5.7%). The former result was somewhat surprising given the difficulty and somewhat subjective nature of the cytotoxin assay. The fact that all three sites perform the cytotoxin assay routinely may account for this observation (i.e., experience and technical skill may be required to achieve a level of consistent

performance with the cytotoxin assay). The low degree of variability in the performance of the ImmunoCard *C. difficile* test may perhaps be attributed to its self-contained test format with objective color change endpoints. The latex agglutination method was intermediate in the variability of its sensitivity characteristics (SD, 12.3%). This method generally requires some experience with reading of latex agglutination endpoints, and interpretation of the results may be subjective with respect to weakly positive samples. The greatest variability in sensitivity parameters occurred with the culture methods (SD, 24.7% for culture and 28.0% for toxigenic culture). This variability was due entirely to a dramatically lower *C. difficile* isolation rate from one site. While this observation lacks ready explanation, it should be noted that successful *C. difficile* recovery by culture is challenging and requires the proper selection and use of media for primary isolation, specialized equipment, and specific confirmatory methods (24). All three sites had extensive experience with *C. difficile* culture. There are reports of medium-dependent recovery variability (23), but this factor was not systematically evaluated in our study.

In conclusion, the study presented here reinforces the challenges and complexity of the laboratory diagnosis of *C. difficile* disease. The new ImmunoCard *C. difficile* test for the GDH of *C. difficile* proved to be a sensitive method for the direct detection of this marker in stool specimens, offering significant improvement over the latex agglutination test. When performance was evaluated with respect to the diagnosis of CDAD, the sensitivity of the ImmunoCard *C. difficile* test for CDAD proved to be equivalent to that of the cytotoxin assay, indicating that it is a useful substitute for or adjunct to current *C. difficile* test methods, especially when rapid results are considered to be of value.

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