JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2001, p. 2846–2849 Vol. 39, No. 8
0095-1137/01/$04.00+0 DOI: 10.1128/JCM.39.8.2846–2849.2001

Evaluation of Methods for Detection of Toxins in Specimens of Feces Submitted for Diagnosis of *Clostridium difficile*-Associated Diarrhea

DON O'CONNOR,[1]* PEARL HYNES,[2] MARTIN CORMICAN,[2,3] EDWARD COLLINS,[2] GERALDINE CORBETT-FEENEY,[2,3] AND MICHAEL CASSIDY[1]

Microbiology Laboratory, Portiuncula Hospital, Ballinasloe, County Galway,[1] and Department of Medical Microbiology, University College Hospital,[2] and Department of Bacteriology, National University of Ireland,[3] Galway, Ireland

Received 21 February 2001/Returned for modification 18 April 2001/Accepted 4 June 2001

***Clostridium difficile* is the principal pathogen associated with hospital-acquired acute diarrheal disease. We have evaluated the performances of six approaches for diagnosis of *C. difficile*-associated diarrhea (CDAD). Consecutive stool specimens ($n = 200$) from 133 patients were examined by cytotoxin assay, by culture of *C. difficile* on cycloserine-cefoxitin-fructose agar, and by toxin detection using four rapid immunoassay systems (Oxoid Toxin A test, ImmunoCard Toxin A test, TechLab Tox A/B II test, and Premier Toxins A&B test). A diagnosis of CDAD was established for 35 (27%) patients (representing 29% of specimens). The adjusted sensitivity and specificity of the methods were, respectively, 98 and 99% for the cytotoxin assay, 54 and 99% for ImmunoCard, 50 and 98% for Oxoid, 79 and 98% for TechLab, 80 and 98% for Premier, and 57 and 100% for culture. The TechLab and Premier assays are acceptable tests for diagnosis of CDAD but are not equivalent to the cytotoxin assay.**

Nosocomial infection with *Clostridium difficile* increases morbidity and mortality among hospitalized patients and places a significant economic burden on health services (3, 7, 18). Early diagnosis is associated with better prognosis (12); therefore; rapid laboratory diagnosis is highly desirable.

The diagnosis of *C. difficile*-associated diarrhea (CDAD) is usually based on clinical features and detection of *C. difficile* toxin. Tissue culture assay is considered the "gold standard" for the demonstration of *C. difficile* toxins in specimens of feces. The technical complexity, slow turnaround time (24 to 48 h), and lack of standardization of the cytotoxin assay are significant limiting factors (11). As a result, a number of commercial products for rapid immunological detection of toxin have been developed. Some products are based on detection of only *C. difficile* toxin A, while others detect both toxin A and toxin B. The practical importance of detection of both toxins is unclear (4, 7). The impetus to detect both toxins may be supported if the suggestion that toxin $A^- B^+$ strains of *C. difficile* are emerging as significant pathogens is validated (1, 13).

In this study, four rapid immunoassays were evaluated for the detection of *C. difficile* toxins in stool specimens and their performances were compared with that of the cytotoxin assay. The rapid assays consisted of two microwell-based enzyme immunoassays that detected both toxin A and toxin B and two chromatographic cassette-based immunoassays that detected toxin A only. The performances of six assay methods (the four immunoassays, the cytotoxin assay, and bacteriological culture) were evaluated with reference to clinical and biological criteria for diagnosis of CDAD.

* Corresponding author. Mailing address: Microbiology Laboratory, Portiuncula Hospital, Ballinasloe, Co. Galway, Ireland. Phone: (353) 0905-48371. Fax: (353) 0905-48363. E-mail: labmanager@portiuncula.com.

MATERIALS AND METHODS

Two hundred consecutive stool specimens (from 133 adult patients) received in the laboratory for routine investigation of *C. difficile* infection were included in the study. Stool specimens were cultured on the day of receipt. The study included specimens that were transported by routine road transport at room temperature from other health care centers. No rejection criteria were applied in respect of interval from collection to receipt of specimens. A filtrate of each stool specimen was also prepared for the cytotoxin assay and stored at −20°C for testing. The remainder of the stool specimen was frozen at −84°C until tested. All specimens were tested within 6 weeks of receipt and were frozen only once.

Specimens were cultured on cycloserine-cefoxitin-fructose agar (Oxoid, Basingstoke, United Kingdom). The final concentrations of cycloserine and cefoxitin were 500 and 16 μg/ml, respectively. The inoculated plates were incubated in an anaerobic chamber for 48 h at 35°C. Presumptive *C. difficile* colonies, characterized by typical colonial morphology and a distinctive odor, were confirmed by Microscreen latex agglutination (Microgen Bioproducts, Camberly, United Kingdom) for a *C. difficile*-specific somatic antigen. The *C. difficile* reference strain ATCC 43953 was used for quality control purposes.

A filter-sterilized, 1:10 dilution of feces was used to inoculate Vero cell monolayers with and without neutralizing *Clostridium sordellii* antitoxin (Pragma Ltd.). Tissue cultures were examined at 24 and at 48 h. Characteristic cytopathic effect (CPE) neutralized by antitoxin was interpreted as a positive result. Where a cytopathic effect was observed with a 1:10 dilution of feces and was not neutralized by antitoxin, the assay was repeated using a higher dilutions (1:40 and 1:100) of feces.

Four commercial systems for immunological detection of *C. difficile* toxin were evaluated. The tests were performed in batches on the same day, after a single thaw of the stored specimens (−84°C) and within six weeks of freezing. Two immunoassays were used to for detection of toxin A in stool specimens: the ImmunoCard Toxin A test (Meridian Diagnostics Inc.) and the C. Difficile Toxin A test (Oxoid Ltd.). The immunoassays used for the detection of both toxin A and toxin B were the C. Difficile TOX A/B II (TechLab, Inc.) and the Premier Toxins A&B tests. All assays were performed and interpreted according to the manufacturers' instructions.

Chart review and clinical assessment. A retrospective chart review was undertaken for any patient for whom the results of all diagnostic techniques were not in agreement. A diagnosis of CDAD was considered established if the patient fulfilled the following four clinical and/or biological criteria (5): (i) diarrhea with more than three loose or watery stools per day for at least 2 days without any other enteric infection documented, (ii) antibiotic use within 6 weeks preceding the onset of diarrhea, (iii) improvement of diarrhea after antibiotic withdrawal or

TABLE 1. Performance of four immunoassays compared with the cytotoxin assay for the detection of *C. difficile* toxins in 200 specimens of feces

Assay method	Result	No. of cytotoxin assay results[a] (n = 200)		Performance characteristics (%)			
		Positive (n = 61)	Negative (n = 139)	Sensitivity	Specificity	PPV[b]	NPV[c]
ImmunoCard Toxin A	Positive	32	0	52	100	100	83
	Negative	29	139				
Oxoid Toxin A	Positive	30	1	49	99	96	82
	Negative	31	138				
TechLab Toxin A/B	Positive	49	1	80	99	97	92
	Negative	12	138				
Premier Toxin A&B	Positive	50	1	82	99	97	93
	Negative	11	138				

[a] Indeterminate results were initially recorded for five (2.5%) specimens, but these were all resolved on retesting of an appropriate dilution of the stool filtrate.
[b] PPV, predictive value of a positive result.
[c] NPV, predictive value of a negative result.

response to oral vancomycin or metronidazole if administered, and (iv) positive result for a stool specimen in one of the six assays.

Analysis of test performance. Test performance was calculated in two ways. In one analysis, the four immunoassays were compared with the cytotoxin assay to determine sensitivity, specificity, and positive and negative predictive values. All six assay methods (the four immunoassays, the cytotoxin assay, and culture) were then evaluated relative to the overall clinical and biological diagnostic assessment to evaluate relative performances in the diagnosis of CDAD.

All statistical calculations were accomplished as described by Bland (6). McNemar's chi-square test, with a correction for discontinuity, was used to detect statistical differences between the results of the assay methods on matched specimens.

RESULTS

Of 200 specimens tested, 69 (34.5%) specimens from 44 (33%) patients were positive by one or more of the six assays methods. Only 16 (8%) specimens were positive by all six methods. A total of 131 (65.5%) specimens were negative by all assay systems. Concordant negative and positive results with the six assay systems were recorded for 147 (73.5%) stool specimens. The performance characteristics of the immunoassays, relative to the cytotoxin assay as the reference method, are presented in Table 1.

Based on the criteria adopted for the study, 32 patient charts required review for clinical evidence of CDAD. As five charts were unavailable or untraceable, results for seven specimens relating to these five patients were excluded in subsequent analysis. Therefore, results of 193 specimens from 128 patients were available for evaluation of the diagnostic systems compared to overall clinical and biological diagnosis. Based on the chart review, false-positive results were recorded for all the toxin detection systems, including the cytotoxin assay (Table 2). Culture was the only investigation that did not yield false-positive results. Overall, a clinical and biological diagnosis of CDAD was established for 35 (27%) patients from whom 56 (29%) specimens were received. The performance characteristics of the cytotoxin assay, culture, and the immunoassays in the diagnosis of CDAD are presented in Table 3.

Statistical analysis (McNemar's test) revealed that the performances of the Premier and TechLab assays in the diagnosis of CDAD were significantly better ($P < 0.01$) than the performance of either the ImmunoCard or the Oxoid assay. The observed difference between the Premier and TechLab assays was not statistically significant ($P = 1$). Stool consistency was recorded for the 200 specimens as follows: 55% liquid, 36.5% unformed, and 8.5% formed. Positive cytotoxin assay results were obtained from all specimen types. Formed stool specimens accounted for three cytotoxin assay-positive tests. These three tests on formed stool related to three patients who had experiences of diarrhea in the days preceding submission of the specimen of formed stool and who met the case definition of CDAD in other respects also.

Repeat specimens accounted for 34% of the tests in this study. Multiple (more than two) positive cytotoxin assay results were recorded for 13 patients. Five patients had three or more specimens positive, and eight patients had two specimens positive.

DISCUSSION

C. difficile is the most common cause of infectious diarrhea in hospitalized patients (18, 20). In our series a final diagnosis of CDAD was established in 27% of patients from whom all appropriate information was available for review. Our results confirm the status of the cytotoxin assay as the gold standard (20) for diagnosis of CDAD, with a sensitivity of 98% and a specificity of 99%. The cytotoxin assay detected an additional 10 true-positive CDAD specimens (n = 56) above the number identified by the best-performing immunoassay. Only two cytotoxin-positive specimens were obtained from patients who did not meet the criteria for a diagnosis of CDAD. In one case the cytotoxin assay and all of the immunoassays were positive; however, the case did not meet the case definition for CDAD

TABLE 2. Summary of results discordant with the clinical and biological diagnosis of CDAD for each test system studied

Assay	No. of specimens determined as:	
	False positive	False negative
Cytotoxin	2	1
ImmunoCard Toxin A	2	26
Oxoid Toxin A	3	28
TechLab Toxin A/B	3	12
Premier Toxin A&B	3	11
Culture	0	24

TABLE 3. Performance of all assay systems for the detection of CDAD using the final diagnosis based on clinical and laboratory criteria as the gold standard

Assay method	Result	Specimens from patients for whom CDAD status was determined to be (n = 193[a]):		Performance characteristics (%)			
		Positive (n = 56)	Negative (n = 137)	Sensitivity	Specificity	PPV[b]	NPV[c]
Cytotoxin	Positive	55	2	98	99	96	99
	Negative	1	135				
ImmunoCard Toxin A	Positive	30	2	54	99	94	84
	Negative	26	135				
Oxoid Toxin A	Positive	28	3	50	98	91	83
	Negative	28	134				
TechLab Toxin A/B	Positive	44	3	79	98	94	92
	Negative	12	134				
Premier Toxin A&B	Positive	45	3	80	98	94	93
	Negative	11	134				
Culture	Positive	32	0	57	100	100	85
	Negative	24	137				

[a] For five patients from whom 7 of 200 specimens were submitted, full clinical details were not accessible. These seven specimens are excluded from the analysis.
[b] PPV, predictive value of a positive result.
[c] NPV, predictive value of a negative result.

because *Campylobacter jejuni* was isolated from the specimen. It is possible that this may represent a case of true mixed infection. The second cytotoxin assay false-positive result related to a patient for whom diarrhea resolved spontaneously after just 2 days and all of the immunoassays were negative. The cytotoxin assay is not ideal, however, as it is labor intensive, tissue culture facilities are required, and the turnaround time is >24 h.

Culture for *C. difficile* is also relatively slow and in our study had limited sensitivity (57%). There are a number of factors that may have contributed to the relatively poor performance of culture in this study. Neither pretreatment of specimens with alcohol shock nor prereduced media were utilized, measures which have been reported to increase the sensitivity of culture (15). Furthermore, the laboratory serves a number of hospital sites and delays in specimen transport may have contributed to the relatively poor performance of culture. In addition to the limited sensitivity noted in our study, diagnosis by culture is also limited by the detection of both nontoxigenic and toxigenic strains of *C. difficile*. The requirement for a 48- to 72-h delay before obtaining a result if confirmation of strain toxigenicity is attempted is also a significant limiting factor (12).

All of the immunoassays evaluated are relatively simple to perform and provide the facility of rapid same-day turnaround time. The Premier Toxin A&B and TechLab Tox A/B II assays provided the best performance characteristics of the four immunoassays studied. The sensitivity for both these systems observed in our study is consistent with that (77 to 83%) reported for recent studies using the updated Premier Toxin A/B assay (M. Campion, A. T. Evangelista, and J. Mortensen, Abstr. 99th Gen. Meet. Am. Soc. Microbiol. 1999, abstr. C-2, p. 105, 1999) and TechLab assays (83 to 100%) (2, 14, 16; Campion et al., Abstr. 99th Gen. Meet. Am. Soc. Microbiol. 1999).

The sensitivity (54%) of the ImmunoCard test observed in this study is considerably below that reported by others (70 to 92%), (8, 19). Likewise, the observed sensitivity of 50% for the Oxoid Toxin A test is lower than that reported (89%) in a recent study (21). The high rate of false-negative results observed in the ImmunoCard test and Oxoid assays in this study (26 and 28, respectively, of 56 specimens determined as CDAD positive) makes these systems unsuitable for use as an isolated test in our patient population.

The superior performances of the TechLab and Premier assays may in part be related to their ability to detect *C. difficile* toxin $A^- B^+$ strains that are nondetectable with toxin A-specific assays. Apart from one Canadian nosocomial outbreak of CDAD attributed to *C. difficile* toxin $A^- B^+$ strains (1, 4), there are limited data available on the likely clinical impact of such strains, and we have no data to indicate if they are a significant factor in our population.

Repeat specimens accounted for 34% of the tests performed in this study. This is similar to the findings of Renshaw et al. (17). With just one exception, repeat specimens were submitted within 1 week of a positive laboratory finding and in many instances within 48 h. It may be appropriate for laboratories to reject repeat specimens from patients who have already tested positive on a recent previous specimen (9). Rejection of formed stool specimens for analysis of CDAD, as suggested by published guidelines, is also appropriate (10, 11).

Rapid and sensitive diagnostic tests for laboratory confirmation of CDAD are important in the current health care environment. Although none of the studied immunoassays matched the sensitivity of the cytotoxin assay, the cytotoxin assay is not practical for many laboratories as it is labor intensive and technically demanding and does not permit same-day reporting of results. Our study suggests that the Premier and TechLab A/B enzyme immunoassays may represent a satisfactory approach to routine testing for evidence of CDAD.

REFERENCES

1. **Al-Barrak, A., J. Embil, B. Dyck, K. Olekson, D. Nicoll, M. Alfa, and A. Kabani.** 1999. An outbreak of toxin A negative, toxin B positive *Clostridium*

difficile-associated diarrhea in a Canadian tertiary-care hospital. Can. Commun. Dis. Rep. **25:**65–69.
2. **Aldeen, W. E., M. Bingham, A. Aiderzada, J. Kucera, S. Jense, and I. Carroll.** 2000. Comparison of the TOX A/B test to a cell culture cytotoxicity for the detection of *Clostridium difficile* in stools. Diagn. Microbiol. Infect. Dis. **36:**211–213.
3. **Alfa, M. J., T. Du, and G. Benda.** 1998. Survey of the incidence of *Clostridium difficile* infection in Canadian hospitals and diagnostic approaches. J. Clin. Microbiol. **36:**2076–2080.
4. **Alfa, M. J., A. Kabani, D. Lyerly, S. Moncrief, L. M. Neville, A. Al-Barrak, G. K. H. Harding, B. Dyck, K. Olekson, and J. M. Embil.** 2000. Characterization of a toxin-A-negative, toxin-B-positive strain of *Clostridium difficile* responsible for a nosocomial outbreak of *Clostridium difficile*-associated diarrhea. J. Clin. Microbiol. **38:**2706–2714.
5. **Barbut, F., C. Kajzer, N. Planas, and J. C. Petit.** 1993. Comparison of three enzyme immunoassays, a cytotoxicity assay, and toxigenic culture for diagnosis of *Clostridium difficile*-associated diarrhea. J. Clin. Microbiol. **31:**963–967.
6. **Bland, M.** 1999. The analysis of cross-tabulations, p. 225–253. *In* M. Bland (ed.), An introduction to medical statistics, 2nd ed. Oxford University, Oxford, United Kingdom.
7. **Communicable Disease Surveillance Centre.** 2000. *Clostridium difficile* in England and Wales. Commun. Dis. Rep. CDR Wkly. **10:**369.
8. **Fedorko, D. P., H. D. Engler, E. M. O'Shaughnessy, E. C. Williams, C. J. Reichelderfer, and W. I. Smith, Jr.** 1999. Evaluation of two rapid assays for detection of *Clostridium difficile* toxin A in stool specimens. J. Clin. Microbiol. **37:**3044–3047.
9. **Fekety, R.** 1997. Guidelines for the diagnosis and management of *Clostridium difficile*-associated diarrhea and colitis. Am. J. Gastroenterol. **92:**739–750.
10. **Gerding, D. N., S. Johnson, L. R. Peterson, M. E. Mulligan, and J. Silva.** 1995. *Clostridium difficile*-associated diarrhea and colitis: SHEA position paper. Infect. Control Hosp. Epidemiol. **16:**459–477.
11. **Johnson, S., and D. N. Gerding.** 1998. *Clostridium difficile*-associated diarrhea: state-of-the-art clinical article. Clin. Infect. Dis. **26:**1027–1036.
12. **Kelly, C. P., and T. J. LaMont.** 1998. *Clostridium difficile* infection. Annu. Rev. Med. **49:**375–390.
13. **Limaye, A. P., D. K. Turgeon, B. T. Cookson, and T. R. Fritsche.** 2000. Pseudomembranous colitis caused by a toxin A^- B^+ strain of *Clostridium difficile*. J. Clin. Microbiol. **38:**1696–1697.
14. **Lyerly, D. M., L. M. Neville, D. T. Evans, J. Fill, S. Allen, W. Greene, R. Sautter, P. Hnatuck, D. J. Torpey, and R. Schwalbe.** 1998. Multicenter evaluation of the *Clostridium difficile* TOX A/B Test. J. Clin. Microbiol. **36:**184–190.
15. **Marler, L. M., J. A. Siders, L. C. Wolters, Y. Pettigrew, B. L. Skitt, and S. D. Allen.** 1992. Comparison of five cultural procedures for isolation of *Clostridium difficile* from stools. J. Clin. Microbiol. **30:**514–516.
16. **Nicholson, G., and M. Jones.** 1999. Laboratory assessment of five enzyme immunoassay *Clostridium difficile* toxin detection kits. Br. J. Biomed. Sci. **56:**204–208.
17. **Renshaw, A. A., J. M. Stelling, and M. H. Doolittle.** 1996. The lack of value of repeated *Clostridium difficile* cytotoxicity assays. Arch. Pathol. Lab. Med. **120:**49–52.
18. **Spencer, R. C.** 1998. Clinical impact and associated costs of *Clostridium difficile*-associated disease. J. Antimicrob. Chemother. **41**(Suppl. C)**:**5–12.
19. **Staneck, J. L., L. S. Weckbach, S. D. Allen, J. A. Siders, P. H. Gilligan, G. Coppit, J. A. Kraft, and D. H. Willis.** 1996. Multicenter evaluation of four methods for *Clostridium difficile* detection: ImmunoCard C. difficile, cytotoxin assay, culture, and latex agglutination. J. Clin. Microbiol. **34:**2718–2721.
20. **Thielman, N. M.** 2000. Antibiotic-associated colitis, p. 1111–1126. *In* G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), Principles and practices of infectious diseases, 5th ed. Churchill Livingstone, Philadelphia, Pa.
21. **Vanpoucke, H., T. De Baere, G. Claeys, M. Vaneechoutte, and G. Verschraegen.** 2001. Evaluation of six commercial assays for the rapid detection of *Clostridium difficile* toxin and/or antigen in stool specimens. Clin. Microbiol. Infect. **7:**55–64.