

Evaluation of a new enzyme immunoassay for *Clostridium difficile* toxin A

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Abstract

Aims—To evaluate a new enzyme immunoassay (EIA) method for detection of *Clostridium difficile* toxin by comparing it to cytotoxicity assay. To investigate the nature of false negative and false positive EIA results by evaluating clinical and therapeutic parameters.

Methods—737 consecutive diarrhoeal specimens collected from patients clinically suspected of having *C difficile* colitis were tested for the presence of *C difficile* toxin by EIA for toxin A and by cytotoxicity assay. Clinical data were evaluated in all cases positive by either method.

Results—With the cytotoxicity assay as a gold standard, the specificity of EIA for toxin detection was 99.3% and the sensitivity was 62.2%. No false negative EIA specimens were obtained from patients already being treated for *C difficile* colitis. Among patients with cytotoxicity positive specimens, those with EIA positive samples had no clinical features distinguishing them from patients with EIA negative samples.

Conclusions—Although specific, the new EIA method directed against toxin A lacks sensitivity compared to cytotoxicity. False negative EIA tests are not associated with concurrent treatment for *C difficile* colitis nor with any specific clinical features examined in our study.

(J Clin Pathol 1997;50:996-1000)

Keywords: *Clostridium difficile* toxin; enzyme immunoassay; cytotoxicity assay

Clostridium difficile is a component of the intestinal microflora that can cause diarrhoea and pseudomembranous colitis following disruption of the normal microbial environment. Pathogenic strains of *C difficile* produce toxin A and toxin B, present together in virtually all cases. Toxin A was originally considered an enterotoxin and was thought to be largely responsible for human disease; toxin B was initially termed a cytotoxin due to its characteristic cytopathic effect when incubated with various cell lines in culture. Recent models of *C difficile* colitis recognise the enteropathic, cytopathic, and probable synergistic effects of both toxins A and B.¹⁻³ Both toxins have been cloned and sequenced; they have been shown to share 64% sequence homology.^{4,5}

The diagnosis of *C difficile* colitis relies on clinical parameters, such as diarrhoea and recent antibiotic use, and laboratory confirmation. There is no consensus on whether detec-

tion of toxin A or toxin B is a more appropriate indicator of a patient's disease status. The most sensitive routine laboratory test is cell culture assay for cytotoxicity, thought to rely largely on the presence of toxin B. Recently, enzyme immunoassays (EIA) directed against both toxins have been developed and evaluated.⁶⁻²¹

EIA directed against toxin A are appealing assays because they test for the toxin traditionally thought most responsible for pathogenicity. They are also more rapid and less expensive than the cytotoxicity assay. However, EIA for the detection of toxin A, although used in some clinical laboratories, have been subject to criticism for a demonstrated lack of correlation with the cytotoxicity assay and other laboratory methods. Most problematic has been the EIA's lack of sensitivity compared to the cytotoxicity assay.⁶⁻¹¹ Clinical and therapeutic parameters possibly related to false negative EIA results have not been studied previously.

In this study, we evaluated a new one hour EIA method for toxin A detection; it is designed for use with an automated analyser and includes a new blocking step designed to eliminate indeterminate results. We also investigated the nature of discrepant results between the EIA for toxin A and the traditional cytotoxicity assay. We hypothesised that toxins A and B are subject to different mechanisms of test interference, perhaps related to identifiable clinical parameters. We speculated that the presence of antibiotics, including standard treatment for *C difficile*, in the specimen may affect the results of the two tests differently.

Materials and methods

SPECIMENS

Specimens included consecutive diarrhoeal stool samples sent to our laboratory for *C difficile* cytotoxicity assay from 6 May to 19 June and from 26 July to 18 September 1996. Included were samples from hospitalised patients in our 750 bed tertiary care hospital and from outpatients at several affiliated clinics. Quality of the stool specimen, if lipaemic or mucous, was recorded. In addition to performing the cytotoxicity assay as is standard in our laboratory, we performed an additional EIA for toxin A on each specimen. All assays were performed on freshly collected stool specimens on the day of receipt unless the specimen was received after 1230, in which case it was refrigerated overnight and tested the next day. Specimens collected on Saturdays after 1230 were refrigerated until Monday. The refrigeration policy was in accordance with the manufacturers' instructions for both assays.

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Accepted for publication 10 October 1997

Table 1 Comparison of EIA and cytotoxicity results

Number of specimens	737
Cytotoxicity positive/EIA positive	38
Cytotoxicity positive/EIA negative	22
Cytotoxicity negative/EIA positive	5
Cytotoxicity negative/EIA negative	672
Sensitivity	62.2%
Specificity	99.3%
Positive predictive value	88%
Negative predictive value	97%

CYTOTOXICITY ASSAY

A dilution of 1.5 ml stool and 1.5 ml *C difficile* buffer (500 ml Dulbecco's phosphate buffered saline, 5 ml Pen-Strep Fungizone (BioWhittaker, Walkersville, Maryland, USA), 5 ml Fungizone (FlowLabs, McLean, Virginia, USA), and 5 ml gentamicin aqueous solution) was vortexed and centrifuged at 1500 ×g for 10 minutes. The emulsified product was filtered. Filtered supernatant (100 µl) was put into 100 µl of buffer and mixed. The mixture was inoculated into a human embryonic lung tissue culture tube (BioWhittaker, Walkersville, Maryland, USA) and incubated for three days, with daily observation for cytopathic effect. For samples showing cytopathic effect, new culture tubes were inoculated with supernatant plus *C difficile* antitoxin (Bartels, Inc, Issaquah, Washington, USA) and monitored for neutralisation. Assays were deemed positive if the cytopathic effect was reversed by neutralisation. Neutralisation testing was not performed on specimens from patients with a previous positive cytotoxicity assay within the past week.

TOXIN A EIA

A 900 µl sample of diluent and 300 µl of thoroughly mixed stool were vortexed until homogeneous then centrifuged for five minutes at a minimum of 12 000 ×g. The supernatant was processed on a Vidas (BioMerieux Vitek, Inc,

Rockland, Massachusetts, USA) analyser in accordance with the manufacturer's instructions for the Vidas *C difficile* Toxin A II Assay. The Vidas analyser was used to perform an automated enzyme linked fluorescence assay using a solid phase receptacle (SPR) coated with polyclonal rabbit antitoxin A antibody. Supernatant, mouse monoclonal antitoxin A antibody, antimouse antibody conjugated with alkaline phosphatase, and 4-methylumbelliferyl phosphate were sequentially cycled in and out of the SPR. A fluorescent product, 4-methylumbelliferone, was catalysed by enzyme remaining bound within the SPR. Fluorescence intensity was measured by the analyser's optical scanner. Reference standards were processed in parallel. For specimens with fluorescence intensity over 10 000 times the reference, the assay was repeated with a blocking step—the supernatant was diluted 1/4 with sample diluent, added to 10 µl of the manufacturer's blocking reagent, and again run on the Vidas analyser. Daily batches of tests were performed in runs that took approximately 1½ hours each. Blocking steps took approximately 1 hour per specimen.

CLINICAL ASSESSMENT

Clinical data from this institution, including all admission notes, discharge instructions and summaries, and pharmacy and laboratory records, were reviewed for all patients with a positive cytotoxicity assay or toxin A EIA.

STATISTICAL ANALYSIS.

Fisher's exact test was used to obtain two sided p values. Confidence intervals (CI) were determined using the approximation of Woolf.

Results

Seven hundred and thirty seven stool samples were tested. Six hundred and seventy two (91%) were negative by both methods and 65 (9%) were positive by one or both methods. The 65 positive tests were samples from 56 patients. Of the 65 tests with positive results, 38 (58%) were positive by both test methods; 22 (34%) were positive by cytotoxicity assay and negative by EIA for toxin A. Five (8%) were negative by cytotoxicity assay and positive by EIA. Using positive cytotoxicity as a gold standard for disease, the sensitivity of the EIA was 62.2%, and the specificity was 99.3% (tables 1 and 2).

The blocking method was required for 22 (3%) of the 737 samples studied. Blocking was performed for eight cytotoxicity positive cases and yielded a positive result in three (sensitivity, 38%). Blocking was performed for 14 cytotoxicity negative cases and yielded a positive result in one specimen (specificity, 93%).

PRIOR AND FOLLOW UP TESTING

Of the 60 samples with positive cytotoxicity tests, 22 (37%) came from patients previously tested by cytotoxicity assay at this hospital; 13 specimens (nine EIA positive and four EIA negative) came from patients who had previous positive cytotoxicity assays. Three of seven (43%) EIA positive samples from patients

Table 2 EIA results in specimens with positive cytotoxicity assays

	Number of specimens		
	EIA positive	EIA negative	p value
Patients previously tested	14/38 (37%)	8/22 (36%)	1.00
Patients with a previous positive cytotoxicity	9/38 (24%)	4/22 (18%)	0.75
Patients with a previous positive cytotoxicity (of those previously tested)	9/14 (64%)	4/8 (50%)	0.66
Patients with a cytotoxicity assay > 1 week previously	7/38 (18%)	7/22 (32%)	0.34
Patients with a positive cytotoxicity > 1 week previously (of those tested > 1 week previously)	3/7 (43%)	3/7 (43%)	1.41
Patients retested in 1996	19/38 (50%)	11/22 (50%)	1.21
Patients with a positive cytotoxicity upon retesting (of those retested in 1996)	8/19 (42%)	3/11 (27%)	0.47
Patients on metronidazole or oral vancomycin at the time of specimen receipt*	3/33 (9%)	0/19 (0%)	0.27
Patients on metronidazole or oral vancomycin ≤ 7 days before specimen receipt*	5/33 (15%)	0/19 (0%)	0.15
Patients on any antibiotic at the time of specimen receipt	20/38 (53%)	12/22 (55%)	1.00
Patients on any antibiotic ≤ 7 days before specimen receipt	26/38 (68%)	15/22 (68%)	1.00
Female patients	17/38 (45%)	11/22 (50%)	0.79
Patients > 60 years old	20/38 (53%)	15/22 (68%)	0.29
Patients admitted with <i>C difficile</i> associated disease	11/38 (29%)	3/22 (14%)	0.22
Specimen refrigerated at least overnight before processing	3/38 (8%)	5/22 (23%)	0.13
Required blocking	3/38 (8%)	5/22 (23%)	0.13
Mucous specimen	4/38 (11%)	1/22 (5%)	0.64
Lipaemic specimen	1/38 (3%)	1/22 (5%)	1.00

*Excluding patients taking metronidazole or oral vancomycin started on the date of specimen receipt.

Table 3 Test results from patients with repeated assays during the study period

3 patients	Cytotoxicity positive/EIA positive ×2
1 patient	Cytotoxicity positive/EIA positive ×2, then cytotoxicity negative/EIA positive
2 patients	Cytotoxicity positive/EIA positive, then cytotoxicity positive/EIA negative
1 patient	Cytotoxicity positive/EIA negative, then cytotoxicity positive/EIA positive
1 patient	Cytotoxicity positive/EIA negative ×2

tested more than one week previously and three of seven EIA negative samples from patients tested more than one week previously were from patients with a prior positive cytotoxicity test.

Retesting, defined as an additional cytotoxicity assay ordered from the same patient later in 1996, was done for 30 (50%) of the specimens with positive cytotoxicity assays during our study period. Nineteen of these samples were EIA positive and 11 were EIA negative. Among the specimens from patients who were later retested, eight of 19 (42%) EIA positive samples and three of 11 (27%) EIA negative samples were from patients who retested positive on their next cytotoxicity assay ($p = 0.47$; 95% CI, 0.39 to 9.70).

Eight (14%) of the 56 patients with specimens positive by either cytotoxicity or EIA assay had additional samples sent for retesting during the study period (table 3).

ANTIBIOTIC USE

Of the 60 samples with positive cytotoxicity assays, eight were from patients receiving metronidazole or oral vancomycin started on the day that the specimen was obtained (empiric therapy for *C difficile* colitis); in these patients, it was unclear from medical records whether the antibiotics were administered before or after specimen collection. Three samples were from patients on either of these drugs concurrent with testing and an additional two samples were from patients who, though not on either drug at the time of the test, had received metronidazole or oral vancomycin during the previous week. No patient received oral bacitracin. Excluding patients started on empiric treatment on the date of specimen receipt, three of 33 EIA positive samples and none of 19 EIA negative samples were from patients on metronidazole or oral vancomycin at the time the sample was received. Five of these 33 EIA positive samples and none of 19 EIA negative samples were from patients on these antibiotics during or within seven days of specimen receipt.

Thirty two of the 60 specimens with positive cytotoxicity tests were from patients on an antibiotic with anti-*C difficile* activity (including macrolides, aminoglycosides, quinolones, and some cephalosporins and penicillins) during the time their sample was received. This included 20 (53%) of the 38 EIA positive specimens and 12 (55%) of the 22 EIA negative specimens. Forty one of the samples with positive cytotoxicity tests were from patients on any antibiotic with anti-*C difficile* activity during or within seven days of specimen receipt. This included 26 (68%) EIA

positive samples and 15 (68%) EIA negative samples.

Two of the five specimens falsely positive by EIA were from patients on antibiotics concurrent with testing; both were on ofloxacin and intravenous vancomycin.

SPECIMEN PROCESSING

Of the 60 samples with positive cytotoxicity assays, eight were refrigerated at least overnight before testing. This included three EIA positive samples and five EIA negative samples. Of these eight cases with a delay between receipt and testing, one was EIA positive immediately, three required blocking, and four were immediately negative; thus, one (3%) of 35 immediately EIA positive samples and seven (28%) of 25 samples not immediately positive were refrigerated ($p = 0.007$; odds ratio, 0.07563; 95% CI, 0.008615 to 0.6639). Only one of the seven samples not immediately EIA positive was from a patient on antibiotics (ofloxacin) at the time of specimen receipt.

Of the cytotoxicity positive/EIA positive samples, four were mucous and one was lipaemic. Of the cytotoxicity positive/EIA negative samples, one was mucous and one was lipaemic.

One of the five specimens falsely positive by EIA was refrigerated overnight.

PATIENT DEMOGRAPHICS

The male:female ratio was 17:21 in patients with cytotoxicity positive/EIA negative specimens and 11:11 in patients with specimens falsely negative by EIA. Patients with cytotoxicity positive/EIA positive specimens had an average age of 61 and patients with false negative specimens had an average age of 64. Among specimens positive by cytotoxicity, six were from outpatients, including two with EIA positive specimens and four with EIA negative specimens. Eleven patients with cytotoxicity positive/EIA positive specimens were admitted with *C difficile* associated disease (as an admitting diagnosis or determined after study to be the cause of admission); three patients with cytotoxicity positive/EIA negative specimens were admitted with *C difficile* associated disease.

Discussion

Our results show that EIA has a high specificity for detecting toxigenic *C difficile* as defined by a positive cytotoxicity assay. The blocking step performed in our study eliminated indeterminate assays problematic in previous studies using this manufacturer's methods.⁷⁻¹¹ Our study demonstrated a high false negative rate of EIA directed against toxin A, as has been found by others.⁶⁻¹¹

Toxins A and B are likely subject to various mechanisms of assay interference. For example, it is possible that detection of toxin A is affected by a patient's own antibodies against *C difficile* toxin,²²⁻²⁴ treatment with synthetic oligosaccharide sequences,^{25, 26} and blood of types known to be agglutinated by toxin A. Cholestyramine and colestipol may,²⁷ and sucralfate may²⁸ or may not²⁹ interfere with

assays for toxin B. When comparing tests aimed at detecting the two different toxins, it seems relevant to assess parameters possibly associated with different modes of interference.

False negative EIA results were most common among patients not on current or recent antibiotic treatment for *C. difficile* colitis. However, this relation was not significant ($p = 0.15$). We had speculated that false negative samples would be associated with concurrent anti-*C. difficile* antibiotic use, due to different degradation rates of the two toxins after inhibition of toxin production in the specimen (as we have observed by adding vancomycin *ex vivo* to *C. difficile* in continuous culture and showing that EIA turns negative before cytotoxicity testing; Vargas and Onderdonk, unpublished observations, 1996), yet no false negative EIA tests occurred among patients on standard therapy for *C. difficile* within the seven days before testing. In our specimens positive by cytotoxicity assay, a delayed interval between specimen receipt and testing was associated with a false negative EIA result or an EIA requiring blocking in all but one case ($p = 0.007$). All except one of these specimens were from patients without concurrent antibiotic use, further refuting our hypothesis that false negative EIA results are caused by antibiotics in the stool. It is relevant that the sensitivity of EIA was not shown to be impaired by concurrent antibiotic treatment for *C. difficile*. Although, at our hospital, testing of specimens from patients already on such antibiotics occurred only in a small minority of patients, this practice is likely to continue, perpetuated by the current admission requirement by some long term care facilities of a negative laboratory test for *C. difficile*.

Patients retested for *C. difficile* associated disease were more commonly positive on their second cytotoxicity assay if they had tested cytotoxicity positive/EIA positive during our study than if they had tested cytotoxicity positive/EIA negative, suggesting that a negative EIA may indicate milder disease or predict a more rapid recovery. However, this interaction was not significant ($p = 0.47$). The rate of previous positive assays longer than one week before specimen receipt, perhaps correlating with increased antitoxin A antibody, was higher among patients with false negative specimens than those with cytotoxicity positive EIA negative specimens. Again this was not significant ($p = 0.34$).

No other clinical features among patients with cytotoxicity positive samples distinguished those with EIA positive samples from those with EIA negative samples. Although higher rates of false negative EIA tests occurred in specimens from women, patients older than 60, and patients admitted for something other than *C. difficile* colitis, as well as in specimens that were blocked, refrigerated, and appeared lipaemic or mucous, these results were not significant.

Study of clinical features distinguishing patients with discrepant from those with concordant EIA and cytotoxicity results was limited by available clinical data. Duration of

diarrhoea and time of specimen collection were not well documented. Confounding factors in the clinical aspects of this study included the possibility that starting empiric *C. difficile* treatment may be a marker for the severity or diagnostic certainty of the disease; also, a history of antibiotic use may be a marker for real disease.

The cytotoxicity assay is an imperfect gold standard for *C. difficile* disease and our sensitivity and specificity results are thus limited. However, because cytotoxicity assay is quite sensitive and specific for the presence of *C. difficile* toxin, we believe that it provides a practical comparison for evaluating the EIA. It is a particularly useful test for segregating patients into groups for analysis of clinical parameters that might affect the detection of toxin A versus toxin B.

We conclude that, though rapid, specific, and free from indeterminate results, the new Vidas method of EIA for toxin A lacks sensitivity compared to cytotoxicity assay. Neither concurrent antibiotic treatment for *C. difficile* nor any clinical feature examined in this study was associated with false negative EIA by this method.

The authors extend their thanks to Ms Marianne Folan for her careful and skilful technical aid. Reagents, use of a Vidas analyser, and support for technical assistance were provided by BioMerieux Vitek, Inc.

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