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 cytotoxins due to its characteristic cytopathic effect when incubated with various cell lines in culture. Recent models of C difficile colitis recognise the enteropathogenic, 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.1–3

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 detection 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.4–21 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.4–11 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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Table 1 Comparison of EIA and cytotoxicity results

<table>
<thead>
<tr>
<th>Description</th>
<th>Number of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytotoxicity positive/EIA positive</td>
<td>14/38 (37%)</td>
</tr>
<tr>
<td>Cytotoxicity positive/EIA negative</td>
<td>8/22 (36%)</td>
</tr>
<tr>
<td>Cytotoxicity negative/EIA positive</td>
<td>9/38 (24%)</td>
</tr>
<tr>
<td>Cytotoxicity negative/EIA negative</td>
<td>4/22 (18%)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>9/14 (64%)</td>
</tr>
<tr>
<td>Specifity</td>
<td>4/8 (50%)</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>7/12 (58%)</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>3/20 (15%)</td>
</tr>
</tbody>
</table>

Table 2 EIA results in specimens with positive cytotoxicity assays

<table>
<thead>
<tr>
<th>Description</th>
<th>Number of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients previously tested</td>
<td>26/38 (68%)</td>
</tr>
<tr>
<td>Patients with a positive cytotoxicity</td>
<td>15/22 (68%)</td>
</tr>
<tr>
<td>Patients with a previous positive cytotoxicity</td>
<td>11/22 (50%)</td>
</tr>
<tr>
<td>Patients with a positive cytotoxicity</td>
<td>5/11 (45%)</td>
</tr>
<tr>
<td>Patients with a previous positive cytotoxicity</td>
<td>2/5 (40%)</td>
</tr>
<tr>
<td>Patients with a positive cytotoxicity</td>
<td>1/2 (50%)</td>
</tr>
<tr>
<td>Patients with a positive cytotoxicity</td>
<td>1/1 (100%)</td>
</tr>
</tbody>
</table>

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%).
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 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 specimens with positive EIA had additional samples sent for retesting during the study period (table 3).

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

<table>
<thead>
<tr>
<th>Patients</th>
<th>Cytotoxicity positive/EIA positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 patients</td>
<td>Cytotoxicity positive/EIA positive x2</td>
</tr>
<tr>
<td>1 patient</td>
<td>Cytotoxicity positive/EIA positive x2, then cytotoxicity negative/EIA positive</td>
</tr>
<tr>
<td>2 patients</td>
<td>Cytotoxicity positive/EIA positive, then cytotoxicity positive/EIA negative</td>
</tr>
<tr>
<td>1 patient</td>
<td>Cytotoxicity positive/EIA negative, then cytotoxicity positive/EIA positive</td>
</tr>
<tr>
<td>1 patient</td>
<td>Cytotoxicity positive/EIA negative x2</td>
</tr>
</tbody>
</table>

**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 bactracin. Excluding patients started on empiric treatment on the day 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, one was 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.1-3 Our study demonstrated a high false negative rate of EIA directed against toxin A, as has been found by others.5-11 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.12-24 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
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