Evaluation of Enzyme Immunoassays to Detect Clostridium difficile Toxin From Anaerobic Stool Culture

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Key Words: Clostridium difficile; Enzyme immunoassay; Cytotoxin; Stool culture

Abstract

Stool culture for Clostridium difficile, while necessary for strain typing and antimicrobial surveillance, cannot determine toxin production. We prospectively tested in triplicate 91 C difficile cultured isolates for toxin production by 2 enzyme immunoassays (EIAs) (Meridian Premier Toxins A&B, Meridian Bioscience, Cincinnati, OH; and TechLab Tox A/B II, TechLab, Blacksburg, VA) and cytotoxin neutralization bioassay (CTN). By CTN, 88% (80/91) were toxigenic. Reproducibility was 93% (85/91) for CTN, 80% (73/91) for Meridian EIA, and 79% (72/91) for TechLab EIA. Compared with CTN, sensitivities were 87.1% and 89.2% for the Meridian and TechLab EIAs, respectively. In an additional 115 stool specimens, CTN detected toxin more frequently from cultured isolates (96/115) than stool (84/115). For C difficile toxin detection from isolates, EIA was less reproducible than CTN. EIA methods can be falsely negative in 10% to 12% of isolates, and these should be tested by CTN or polymerase chain reaction. When positive, EIA is fast and reliable for detecting C difficile toxin from culture.

Diagnostic uncertainty exists about the optimal approach to diagnose Clostridium difficile–associated disease.1–3 Physicians seek the most sensitive and specific test to detect C difficile toxin that also yields a rapid result, but the emergence of more virulent strains has promoted a need for cultured isolates, which has complicated testing algorithms.4,5 Historically, enzyme immunoassays (EIAs) for toxins A and B have been popular for laboratory practice and clinical care because the tests are simple to perform and specific, and results are provided within 24 hours. Today, anaerobic stool culture has reemerged as an important test in the diagnosis and management of C difficile infection, remaining the most sensitive test available with the added benefit of providing a clinical isolate that can be used to monitor antimicrobial susceptibility patterns, develop vaccines, and perform strain typing. Culturing stool, however, is not specific because it does not distinguish toxin-producing from non–toxin-producing strains. Some laboratories that have the technical expertise offer a cell culture cytotoxin neutralization bioassay (CTN), a highly specific test to confirm the presence of toxin B–producing strains from direct specimen or culture, but this method is labor-intensive, and results can be delayed up to 48 hours.

During the last 5 years, we observed a more than 60% increase in test requests specifically to recover C difficile from anaerobic stool culture with slightly decreased numbers of requests for CTN. This observation prompted us to explore an efficient, inexpensive, and accurate testing algorithm that incorporates the ability to recover C difficile from stool culture and determine toxin production. While a few laboratories have reported using EIA directly from cultured isolates,7–10 to our knowledge, no studies have specifically
examined its performance in this setting. We compared the performance characteristics of 2 commercial enzyme-labeled immunosorbent assays with cell cytotoxin neutralization bioassay to detect *C difficile* toxin from culture.

**Materials and Methods**

**Clinical Specimens**

*C difficile* isolates that were recovered from stool samples submitted to ARUP Laboratories, Salt Lake City, UT, specifically for *C difficile* culture were collected prospectively. Specimens were collected for comparative analysis by EIA and CTN between May and September 2006, and for comparative analysis by CTN on stool vs CTN on cultured isolates between November 2007 and March 2008. Fecal specimens were received frozen, or, if refrigerated, the specimen was processed if received within 48 hours of collection. Specimens were stored at 2°C to 8°C after initial processing.

**Culture**

All stool samples were plated to cycloserine-cefoxitin-fructose agar with horse blood media by a standard laboratory protocol. *C difficile* was identified based on growth and appearance on cycloserine-cefoxitin-fructose agar with horse blood, Gram stain, fluorescence under a Wood lamp, and the characteristic “horse stable” odor.

**Enzyme Immunoassay**

We used 4 to 6 mature colonies of *C difficile* to prepare suspensions with each EIA kit-specific diluent, and they were tested for toxin with Premier Toxins A&B (Meridian Bioscience, Cincinnati, OH) and *C difficile* Tox A/B II (TechLab, Blacksburg, VA) kits using the manufacturers’ protocols for direct specimen testing. Toxin A and toxin B were not distinguished with these test methods.

**Cytotoxin Neutralization Bioassay**

CTN was performed on all *C difficile* isolates in this study. Colony suspensions were prepared with phosphate-buffered saline. For testing of fecal specimens, patient stool was diluted 1:10, centrifuged, and, if necessary, filtered to clarify the supernatant. *C difficile* suspensions or fecal supernatants from each patient were inoculated into 2 wells of human foreskin fibroblast plate in 2% minimum essential medium diluent containing penicillin, streptomycin, and amphotericin B (Fungizone). *C difficile* goat antitoxin (TechLab) was added to one of the wells. Plates were incubated at 37°C in 5% carbon dioxide and examined at 4, 24, and 48 hours for cytopathic effect (CPE) including rounding of cells and disruption of cell monolayer. The presence of CPE in the sample-only well and the absence of CPE in the sample well with antitoxin was defined as a positive result.

**Data Analysis**

For comparison of EIA and CTN, all toxin testing was performed in triplicate for each test method evaluated. A positive toxin result for each test method was defined as having at least 2 positive test results by that method with each isolate. When all results tested in triplicate within a test method agreed, the test result for that isolate was considered reproducible. For comparative analysis, CTN was considered the “gold standard.” Sensitivity, specificity, negative predictive value, and positive predictive value with 95% confidence intervals for EIA were determined. Statistical analyses were performed with Bayesian calculations. The binomial test was used for comparison of reproducibility between EIA and CTN.

**Results**

For comparative analysis of EIA and CTN on *C difficile* isolates, 91 *C difficile* isolates were recovered from stool culture representing 91 patients. CTN, EIA-Meridian, and EIA-TechLab identified 80 and 11, 70 and 21, 71 and 20 toxin-producing and non–toxin-producing strains, respectively. The performance characteristics of the EIAs using CTN as the gold standard are given in Table 1. Both EIA methods performed similarly with less than 90% sensitivity and specificity of 97% or more. Sensitivity, specificity, negative predictive value, and positive predictive value were not significantly different between the 2 EIAs. When we evaluated the reliability of each test method, CTN was significantly more reproducible than the EIA-Meridian and EIA-TechLab methods, with a P value of less than .05 compared with either EIA Table 2. We obtained 115 samples for comparison of CTN on stool and CTN on colonies. Both assays were positive in 82 (71.3%) of 115 cases and negative in 17 (14.8%). CTN on colonies was positive in 14 cases in which stool CTN was negative. CTN on stool was positive in 2 cases in which CTN on colonies was negative.

**Discussion**

Standardization for diagnosis of *C difficile*–associated enteric disease is essential for hospital institutions and epidemiologists to accurately conduct surveillance and effectively implement infection control measures. Anaerobic stool culture for *C difficile* remains a valuable laboratory test. With the emergence of epidemic, highly virulent strains, the role of culture has increased in importance because it provides an isolate for further analysis such as strain typing or susceptibility
testing. EIA can serve as a fast, inexpensive, and preliminary method to detect *C difficile* toxin from culture, and, when positive, the likelihood of having a toxin-producing strain is high in settings with high disease prevalence.

The performance of EIA on cultured isolates in this study was within the range of previously reported results from testing directly on stool specimens. The TechLab EIA has reported sensitivities of 84% to 96% and specificities of 87% to 100%8,12,15 while the Premier Toxins A&B EIA has reported sensitivities of 60% to 97% and specificities of 93% to 100%.3,14,16,17 In our study, both EIA kits tested had specificities of 97% or more and positive predictive values of 99.5% or more when using the CTN assay on cultured isolates as the gold standard. These assays, therefore, would be convenient as screening tools for toxin detection in *C difficile* isolates. A systematic examination of the reproducibility of EIA has not been performed before this study, and clinical microbiologists should be aware that EIA testing from culture is only 80% reproducible. Based on these data, we propose a 2-step algorithm for the detection of toxin from *C difficile* isolates recovered from anaerobic stool culture. When the EIA result is positive, the result can be interpreted as a true positive. When the EIA is negative, *C difficile* isolates should be tested by CTN or nucleic-acid amplification methods (when available) to more accurately and reliably exclude *C difficile*-associated enteric disease.

Lability of *C difficile* toxin may offer an explanation for the approximately 10% false-negative EIA results in which previous data suggest that the microorganism might require certain environmental conditions, such as stool, to elaborate toxin.18 Isolates were not serially subcultured, and suspensions were prepared specifically to provide the optimal environment to detect toxin. Despite our efforts to minimize these variables, EIA reproducibility from cultured isolates still remained poor, suggesting that testing algorithms with EIA may need revision. We also confirm previous findings that toxin production is not diminished after culture of *C difficile* isolates on solid media, but rather was enhanced in a number of isolates. In fact, in vitro testing of *C difficile* isolates for toxin production may be more sensitive than toxin testing directly from stool,7,10,19,20 a finding that may be related to higher organism load enhancing toxin detection.7

We acknowledge that this study had a few limitations. Our observation that EIA had poor reproducibility and yet retained high positive predictive value reflects the large numbers of toxin-producing strains compared with non–toxin-producing strains in this study. In addition, no attempt was made to discriminate between toxin A and toxin B. We do not believe this limitation impacted the results of our study because there were no cases in which the CTN result was negative and the EIA result was positive. Also, human disease from toxin A–positive/toxin B–negative *C difficile* has not been documented. Another potential limitation is that we did not use a third method, specifically nucleic-acid amplification, to analyze discrepant results between EIA and CTN.

EIA can serve as a quick screening tool for toxin production from cultured isolates. A negative result, however, cannot reliably rule out the presence of toxin. Also, EIA was poorly reproducible, and a single EIA should not be solely relied on for confirmation of toxin production from anaerobic culture, particularly in clinical contexts with low prevalence of toxin-producing strains.

![Table](https://example.com/table1.png)

**Table 1**

<table>
<thead>
<tr>
<th>Test Method</th>
<th>No. of Tests</th>
<th>Performance (95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>CTN</td>
<td>240</td>
<td>33</td>
</tr>
<tr>
<td>EIA (Meridian)</td>
<td>209</td>
<td>33</td>
</tr>
<tr>
<td>EIA (TechLab)</td>
<td>214</td>
<td>32</td>
</tr>
</tbody>
</table>

*CTN, cytotoxin neutralization bioassay; EIA, enzyme immunoassay; NPV, negative predictive value; PPV, positive predictive value.

* CTN served as the “gold standard.” Meridian Bioscience, Cincinnati, OH; TechLab, Blacksburg, VA.

![Table](https://example.com/table2.png)

**Table 2**

<table>
<thead>
<tr>
<th>Test Method</th>
<th>No. of Isolates (n = 91)</th>
<th>Reproducibility (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTN</td>
<td>85 (93)</td>
<td>—</td>
<td>.01</td>
</tr>
<tr>
<td>EIA (Meridian Bioscience, Cincinnati, OH)</td>
<td>73 (80)</td>
<td>.016</td>
<td></td>
</tr>
<tr>
<td>EIA (TechLab, Blacksburg, VA)</td>
<td>72 (79)</td>
<td>.01</td>
<td></td>
</tr>
</tbody>
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*CTN, cytotoxin neutralization bioassay.*

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References


