Detection of *Clostridium difficile* toxin:
Comparison of enzyme immunoassays
with results obtained by cytotoxicity assay

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**Abstract**

Several kinds of laboratory techniques are available to detect *C. difficile* toxin in fecal samples. Because questions have been raised about the reliability of immunoassays compared to the accepted standard, cytotoxicity, we studied three EIAs and one rapid EIA, demonstrating relatively good sensitivity and specificity when compared to cytotoxicity.

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*Clostridium difficile* colitis has increased in prevalence and severity in hospitals throughout the developed world (4, 7, 10). Prompt, accurate diagnosis and early treatment shorten the duration of diarrhea which, in turn, further reduces spread of infection (6, 12, 14). A cytotoxicity assay (CYTA) has been regarded as the “gold standard” for detecting *C. difficile* toxin in a fecal sample (5), but this test is labor-intensive and requires 18-48 hr incubation before a final reading can be made. We compared CYTA with commercially available enzyme immunoassays (EIA) and one rapid EIA card immunoassay, all of which detect *C. difficile* toxins A and B.

CYTA (*C. difficile* Toxin Detection Kit, Diagnostic Hybrids, Athens, OH) utilized microwell plates containing cultured human foreskin fibroblasts to detect toxin B. Every study included a toxin-positive and a toxin-negative control, and each sample was studied without or with the addition of antibody to toxins A and B. Results were read after overnight incubation (16-18 hr) and again after 48 hr incubation by readers who were blinded as to the results of other studies. For EIA, we initially used Premier™ Toxins A&B EIA kit (EIAPrem; Meridian
Bioscience, Cincinnati, OH) in accord with the manufacturer’s instructions to
detect *C. difficile* toxins A and B. Subsequent studies utilized *C. DIFFICILE TOX*
*A/B II™* (EIATech; TechLab, Blacksburg, VA) and ProSpecT® Clostridium
difficile Toxin A/B Microplate Assay (EIAPrem; Remel, Lanexa, KS), both of which
also detect toxins A and B of *C. difficile*. One rapid immunochemical detection
.card (REIA; ImmunoCard®, Meridian Bioscience) was also tested in accord with
the manufacturer’s instructions; each card included positive and negative
controls.

In the first phase of this study, we addressed the question of whether EIA
or REIA could reliably reproduce results of CYTA, using a single representative
EIA and REIA. Accordingly, we initially tested 446 consecutive fecal samples
submitted to the Microbiology Laboratory, Michael E. DeBakey VA Medical
Center, Houston, for detection of *C. difficile* toxin, comparing CYTA, EIAPrem
and REIA. For the purposes of this study, CYTA was regarded as providing a
true result. Seventy-six (17.0%) samples were positive by CYTA. In every case
in which the result was positive, a correct reading could be made after overnight
incubation (16-18 hr), although the manufacturer’s instructions recommend
incubation for up to 48 hr incubation for a final reading. As shown in Table 1, of
these 76 samples, 75 were also positive by EIAPrem (sensitivity, 98.7%, CI 92-
99, Microsoft® Excel 2003). Of the 370 that were negative by CYTA, 10 were
positive by EIAPrem (specificity, 97.3%, CI 95-98). Thus, for EIAPrem, the
positive predictive value was 75/85 (88.2%, CI 79-94) and the negative predictive
value, 360/361 (99.7%, CI 98-99).
REIA was positive in 73 of 76 CYTA-positive specimens (Table 1; sensitivity, 96.1%, CI 88-99). Of the 370 CYTA-negative specimens, 4 were positive by REIA (specificity, 98.9%, CI 97-99). For REIA, the positive predictive value was 73/77 (94.8%, CI87-98), and the negative predictive value was 366/369 (99.2%, CI 97-99).

Having shown that results obtained with a representative EIA much more closely resembled those of CYTA than had been suggested by some earlier literature (2, 9, 13, 15, 16), we then compared 3 EIAs that are commercially available in the United States, utilizing receiver operator curve statistical analysis (True Epistat; Richardson, TX). For this phase of the study, we used a convenience sample of 131 fresh fecal specimens, 54 of which were CYTA-positive and 77 of which were CYTA-negative. As shown in Table 2, sensitivity of EIAPrem and EIATech were each 96.3%; sensitivity of EIAPro was 90.7%. There were no statistical differences seen when comparing test sensitivities of EIAPrem or EIAPro and EIATech (p = 0.25). Specificity of EIAPro was 97.4%, vs 93.5% for EIAPrem and 87.0% for EIATech (Table 2). Although there was no difference in test specificities between EIAPro and EIAPrem (p=0.45), the specificity of EIAPro was significantly lower than that of EIATech (p = 0.04)

The results of this study show that, when compared to CYTA, three commercially available EIAs and one REIA reliably detect the presence of *C. difficile* toxin in fecal samples. When EIAPrem and REIA were compared in 446 samples, 431 (96.6%) were either positive or negative in all three assays, indicating a high degree of concordance. When EIAPrem, EIATech and EIAPro
were compared, the overall concordance among all three tests was somewhat lower (112 of 131, 85.5%) because of the relatively lower sensitivity but higher specificity of EIAPro. We repeated all tests on every sample that yielded discordant results, leading to a marginally better rate of concordance, but data presented in this paper were those determined by the initial test, just as would be the case for data provided to clinicians by clinical laboratories.

Earlier studies of EIA for toxins A and B reported sensitivities of 57% to 100%, averaging 83% when compared to CYTA (1-3, 8, 9, 11, 13, 15-17). Many of these studies required that three samples be tested, and a positive result was reported if any of the three was positive. The important implication of our results is that, using kits that are presently available, a single EIA is likely to yield a highly reliable result; multiple samples need not be submitted for analysis, and only modest benefits in diagnostic accuracy would be obtained by replacing EIA with CYTA.

It is worth noting that, in this study, when discordant results were obtained, they were generally confirmed by repeat testing. In those few instances when repeat testing yielded a different result, we still calculated our data based on the initial one in order to simulate the usual clinical situation. Interestingly, results in one case illustrate the adage that there is no true “gold standard.” One fecal sample that was positive by CYTA was negative by EIA and REIA; the three assays were repeated, yielding the same results. A review of this patient’s records showed that he had not received antibiotics and had no clinical findings of *C. difficile* associated disease (no diarrhea, fever, leukocytosis or abdominal
pain); in fact, the primary physicians were unclear why a specimen had been submitted. A new fecal sample from this patient was negative in all assays. The patient received no treatment for *C. difficile* colitis and remained free of symptoms. We regard the initial CYTA result as being falsely positive.

CYTA, the ‘gold standard’ for assaying toxins A and B of *C. difficile*, is labor-intensive, requires tissue-cultured cells and an inverted microscope, and needs overnight incubation before reading. EIA is also labor intensive, requiring several hours of technician time and an EIA reader; batching of specimens increases cost-efficiency but may delay reporting of results, especially if tests are not done every day. REIA is more costly for each test but, for laboratories that process only occasional samples, appears to provide prompt, reliable and cost-effective results.
Acknowledgments / Conflicts of Interest

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Table 1

Cytotoxicity, enzyme immunoassay\(^a\) and rapid enzyme immunoassay\(^b\) for detecting \textit{C. difficile} toxin in 446 consecutive fecal samples

<table>
<thead>
<tr>
<th>n=446</th>
<th>CYTA(^a)</th>
<th>EIA(^b)</th>
<th>REIA(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>73</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>+</td>
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<td>-</td>
<td>+</td>
</tr>
<tr>
<td>358</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

Sensitivity (relative to CYTA) 98.7% 96.1%
Specificity (relative to CYTA) 97.3% 98.9%

\(^a\)CYTA, cytotoxicity assay

\(^b\)EIA, Premier\textsuperscript{TM} Toxins A&B enzyme-linked immunoassay

\(^c\)REIA, ImmunoCard\textsuperscript{®} rapid card enzyme-linked immunoassay
Table 2

Comparison of 3 enzyme immunoassays for *C. difficile* toxin in a convenience sample of 131 fecal specimens

<table>
<thead>
<tr>
<th>n=131</th>
<th>CYTA(^a)</th>
<th>EIAPrem(^b)</th>
<th>EIATech(^c)</th>
<th>EIAPro(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>49</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>2</td>
<td>+</td>
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<td>+</td>
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<td>-</td>
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<td>-</td>
<td>+</td>
</tr>
<tr>
<td>63</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

- Sensitivity (vs CYTA) 96.3%  96.3%  90.7%
- Specificity (vs CYTA) 93.5%  87.0%  97.4%

\(^a\) CYTA, cytotoxicity assay

\(^b\) EIAPrem = Premier\(^TM\) Toxins A&B enzyme-linked immunoassay

\(^c\) EIATech = *C. DIFFICILE TOX A/B II*\(^TM\)

\(^d\) EIAPro = ProSpecT® Clostridium difficile Toxin A/B Microplate Assay