NOTES

Comparison of the Premier Toxin A and B Assay and the TOX A/B II Assay for Diagnosis of *Clostridium difficile* Infection

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*Clostridium difficile* causes nosocomial diarrhea and is responsible for complications such as pseudomembranous colitis, megacolon, and perforation. Using 442 stool specimens, we compared the sensitivities and specificities of the Premier toxin A and B (Meridian Bioscience, Inc.) and *C. difficile* TOX A/B II (TechLab, Inc., Blacksburg, VA) immunoassays in the Virology Department of the Kaiser Permanente Regional Reference Laboratories. The Premier toxin A and B assay demonstrated a higher sensitivity (97.44%) and a higher positive predictive value (79.17%) than the *C. difficile* TOX A/B II assay (87.18% and 75.56%, respectively), while assay specificities and negative predictive values were similar. We also performed experiments using serially diluted, purified toxin A and B antigens to understand the basis for assay differences. The two assays’ toxin A antibodies detected toxin A at comparable levels. Preliminary results indicated that the toxin B antibody in the Premier toxin A and B assay could detect toxin B at a concentration of 125 pg/100 μl, while the toxin B antibody in the *C. difficile* TOX A/B II assay could not detect toxin B below a concentration of 250 pg/100 μl. Therefore, the Premier toxin A and B assay provides greater sensitivity than the *C. difficile* TOX A/B II assay, perhaps due to a superior detection ability of its toxin B antibody.

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*Clostridium difficile*, the primary agent causing nosocomial diarrhea, affects 300,000 to 3,000,000 hospital patients in the United States each year at a cost of 1.1 billion dollars (6, 8, 13). An estimated 20% of all hospital inpatients and 25 to 80% of all healthy newborns and infants test positive for *C. difficile*. Virtually all (95 to 100%) cases of pseudomembranous colitis are attributed to *C. difficile* infection (6, 9). Many infected individuals remain asymptomatic, but symptomatic individuals may present with mild diarrhea, high fever, severe abdominal pain, colitis, prolonged ileus, or perforation (6, 13).

At greatest risk for *C. difficile*-associated diarrhea (CDAD) are hospitalized adults older than 65 years and patients with certain underlying conditions (6, 15). Most CDAD cases resolve within several days if diagnosed promptly and treated appropriately (14). However, relapse occurs in 20% of patients, and those individuals are still at risk for subsequent relapses and development of severe complications (13).

All toxigenic and nontoxigenic *C. difficile* strains synthesize glutamate dehydrogenase (GDH), but only toxigenic strains produce toxin A and toxin B, virulence factors thought to function synergistically (10, 11, 16). Aberrant strains may express additional virulence factors, such as binary toxin, which is similar to the iota toxin of *Clostridium perfringens* and *Clostridium spiroforme*. Binary toxin has been identified among a low percentage of clinical isolates. However, the role of this toxin in CDAD is not clear at the present time (18).

Once it became clear that *C. difficile* can cause disease, numerous diagnostic techniques were developed, but the cytotoxin B assay has been considered the gold standard, with high sensitivity (94% to 100%) and specificity (99%) (3, 5, 6, 13, 17, 18). Two currently popular diagnostic systems for detecting *C. difficile* are based on detecting the GDH common antigen (2) or toxin A and/or toxin B. Generally, diagnosis based on detecting toxins is superior, since GDH cannot distinguish toxigenic strains from nontoxigenic strains. Because of evidence for strains with unusual toxin profiles, including polymorphic A and B genes, diagnosis using tools that identify both toxins A and B is essential. Assays designed for detecting only one toxin have missed detecting virulent *C. difficile* strains possessing aberrant profiles (1, 4, 12, 19). Because rapid diagnosis and treatment predict a high cure rate, the current medical approach stresses rapid intervention, using aggressive evaluation to detect *C. difficile* infection (7).

Toxin detection tests are the choice of most clinical laboratories and include simple and fast commercially available immunoassays or enzyme-linked immunosorbent assays (ELISAs). Most ELISAs detect 1 to 2 ng/ml of toxin A and/or B in stool, are sensitive (49% to 80% for toxin A only and 71% to 94% for both toxins) (10, 13), and are specific (92% to 98%). O’Connor et al. (14) found that the Premier toxin A and B and *C. difficile* TOX A/B II assays had sensitivities of 80% and 79% and specificities of 98% and 98%, respectively.

We undertook a prospective study in the Virology Department of the Kaiser Permanente Regional Reference Laboratories (RRL) to compare the performances of these two frequently used immunoassays that test for both toxins. While we had routinely been using the *C. difficile* TOX A/B II assay for diagnosing CDAD in clinical specimens, we had encountered...
difficulties or inefficiencies with this assay, including limited reproducibility and excessive repeat analysis. The manufacturer requires repeat testing of all low-positive results (optical density [OD] of less than 0.200) that are adjacent to specimens that yield high-positive values (C. difficile TOX A/B II product insert; TechLab, Inc.). The Premier toxin A and B assay does not require repeat evaluation for low-positive results and has greater sensitivity than, and equal specificity to, the C. difficile TOX A/B II assay (2001 Premier toxin A and B product insert; Meridian Bioscience, Inc.).

Patient population. The testing population for this study consisted of 442 new and previously unevaluated stool specimens. Samples were collected in 2002 in Southern California from children and adults, with ages ranging from 10 months to 93 years, as soft or liquid fresh stool in sterile cups without preservatives and transported at 4°C to the Kaiser Permanente RRL. Specimens were excluded if they were unable to be tested by both methods due to insufficient volumes.

Sample handling and storage. The specimens were assayed as they arrived at Kaiser Permanente RRL in North Hollywood, CA. The samples were assayed the same day without replication in nine matched batches with the Premier toxin A and B ELISA and the C. difficile TOX A/B II assay. Between runs, the specimens were stored at 4°C. The Premier toxin A and B and C. difficile TOX A/B assay results were separately recorded without knowledge of the other assay’s results.

Enzyme immunoassays for detecting toxins A and B. The microwell ELISAs used for detecting toxins A and B in stool samples were the Premier toxin A and B (Meridian Bioscience, Inc., Cincinnati, OH) and the C. difficile TOX A/B II (TechLab, Inc., Blacksburg, VA) assays. Assays were performed according to the manufacturers’ instructions. Positive results for the Premier toxin A and B assay are indicated by OD values at 450/630 nm (OD_{450/630}) of 0.100, and negative results are indicated by OD_{450/630} values of <0.100. Positive results for the C. difficile TOX A/B II assay are indicated by OD_{450/630} values of 0.080, and negative results are indicated by OD_{450/630} values of <0.080. The quality control section of the C. difficile TOX A/B II product insert also states the caveat that “a sample that yields a weak positive result (i.e., <0.200) and is adjacent to a strong positive should be repeated to assure carryover did not occur.” The quality control section of the Premier toxin A and B product insert does not require repeat testing of low-positive results.

Cytotoxin assay for resolving discordant results. Samples that yielded discordant results between the assays were resolved at the Good Samaritan (Tri-Health) Hospital Laboratories, Cincinnati, OH, using the cytotoxin B tissue culture assay (Bartels Immunodiagnostics Supplies, Bellevue, WA) according to the manufacturer’s instructions. Discordant samples were frozen at −80°C immediately following ELISA in the Kaiser Permanente RRL and then batched weekly and shipped overnight on dry ice to Tri-Health Laboratories for cytotoxin resolution.

Intrarun and interrun comparisons. Samples were also compared for intrarun and interrun precision by testing, with the Premier toxin A and B and C. difficile TOX A/B II assays, specimens “X” (a rotavirus-positive, C. difficile toxin-negative specimen), “Y” (a C. difficile toxin-positive specimen), and “Z” (a C. difficile toxin-negative specimen). These archived samples had been previously evaluated for rotavirus with the Premier

| Positive  | Negative |
|-----------|----------|
| 33        | 12^b     |
| 15^a      | 382      |

^a False positives. ^b False negatives.

Rotaclove assay (Meridian Bioscience, Inc.) and for C. difficile with the C. difficile TOX A/B II assay as standards. The intrarun precision using the X, Y, and Z specimens was assayed five times within one ELISA run, and the interrun precision using the same X, Y, and Z specimens was assayed along with the 442 test specimens during eight of nine ELISA runs.

Testing of detection limits for antitoxin A and antitoxin B antibodies. Toxin A and toxin B, each at a concentration of 1 ng/100 μl, were serially diluted in the Premier toxin A and B and C. difficile TOX A/B II sample diluents to 250-μl volumes. The assays were performed as described above without replication, according to each manufacturer’s instructions, using the substituted toxin dilutions. The assays were carried to completion in order to compare the detection limits of each kit’s antitoxin A and antitoxin B antibodies.

Statistical analysis. Differences between the cytotoxin assay experiments, performed for resolving discordant results between the two enzyme immunoassays, were analyzed using PROC FREQ, an exact binomial test (SAS system). The vari-ances, standard errors, and confidence intervals for sensitivity and specificity differences were evaluated using the Simple Interactive Statistical Analysis for diagnostics (http://home.clara.net/sisa/binomial.htm).

Assay data. Following testing for the presence of C. difficile toxins A and B in 442 patient specimens, initial data distribution indicated that 7.47% of the specimens were true positives, 86.43% of the specimens were true negatives, 3.39% were false negatives, and 2.71% were false positives. We define a true-positive test result as a test result that was positive by both the Premier toxin A and B and C. difficile TOX A/B II assays, and we define a true-negative test result as a test result that was negative by both assays. Table 1 summarizes these initial data.

Cytotoxin B assay resolution of discordant results indicated that 6.11% of the specimens were discordant. After resolution and recategorization of the discordant specimens, the Premier toxin A and B assay detected 38 out of 39 toxin B-positive specimens, while the C. difficile TOX A/B II assay detected 34 out of 39 toxin B-positive specimens. Therefore, 6 out of 39 (15.38%) positive specimens remained discordant after cytotoxin resolution.

The predictive values of the tests were based on prevalence and test sensitivity and specificity. The overall prevalence of C. difficile toxins A and B was 8.82%, as determined by the immunoassays and the cytotoxin assay. We defined sensitivity as the number of true positives divided by the sum of the number of true positives and the number of false negatives; specificity as the number of true negatives divided by the sum of the number of true negatives and the number of false positives;
positive predictive value (PPV) as the number of true positives divided by the sum of the number of true positives and the number of false positives; and negative predictive value (NPV) as the number of true negatives divided by the sum of the number of true negatives and the number of false negatives. The formula for PPV predicts how well a test will correctly determine positive results compared to all true test positives, and conversely, the NPV predicts how well the test will correctly determine negative results compared to all true test negatives. The resolution of discordant results is summarized in Table 2.

Assay sensitivity. The Premier toxin A and B assay was more sensitive than the C. difficile TOX A/B assay for detection of C. difficile toxins in the stool specimens (P < 0.10). The variances, standard errors, and 95% confidence intervals for sensitivity data of the Premier toxin A and B and TechLab C. difficile TOX A/B II assays were, respectively, as follows: (i) 0.00064 and 0.00287, (ii) 0.02531 and 0.05353, and (iii) 0.925 to 1.024

Assay specificity. Neither the Premier toxin A and B assay nor the C. difficile TOX A/B II assay was significantly more specific than the other for detection of C. difficile toxins in stool (P < 1.0). The variances, standard errors, and 95% confidence intervals for specificity data of the Premier toxin A and B and C. difficile TOX A/B II assays were, respectively, as follows: (i) 0.00006, and 0.00007, (ii) 0.00775 and 0.00812, and (iii) 0.96 to 0.977.

Assay PPV. The Premier toxin A and B assay had a higher PPV than the C. difficile TOX A/B II assay for detection of C. difficile toxins in stool, as predicted by the greater sensitivity of the Premier toxin A and B assay (P < 0.10).

Assay NPV. Neither the Premier toxin A and B assay nor the C. difficile TOX A/B II assay yielded 25% false positives for the other (P < 1.0).

Intrarun comparison using X, Y, and Z specimens. The Premier toxin A and B assay correctly indicated negative results in all eight runs for specimens X, Y, and Z, whereas the C. difficile TOX A/B II assay yielded 25% false positives for specimen X and 100% accurate results for specimens Y and Z. Table 3 summarizes these results.

Intrarun comparison using X, Y, and Z specimens. Both the Premier toxin A and B and C. difficile TOX A/B II assays correctly yielded negative results for all five out of five replicates within the intrarun test for specimens X, Y, and Z (data not shown).

Testing of detection limits for antitoxin A and antitoxin B antibodies. The results from experiments performed using diluted toxins A and B in place of samples indicated that both assays’ antitoxin A antibodies detected toxin A at a concentration of 250 pg/100 μl. Results at a concentration of 125 pg/100 μl were similar between the Premier toxin A and B and the C. difficile TOX A/B II assays. Although test criteria indicated that the Premier toxin A and B result at a concentration of 125 pg/100 μl was negative, the C. difficile TOX A/B II result at that toxin concentration was considered positive. Results are summarized in Table 4.

The Premier toxin A and B assay’s antitoxin B antibody detected toxin B with strong positive values at concentrations of 500 pg/100 μl, 250 pg/100 μl, and 125 pg/100 μl. The C. difficile TOX A/B II assay detected toxin B with comparatively weaker signals at every dilution that declined into negative results at concentrations below 250 pg/100 μl. Table 5 summarizes these results.

Discussion. Clostridium difficile infection is a serious and costly medical problem. This pathogen affects a large number of patients in the United States alone and is persistent in the environment, colonizing directly or through its robust spores. These issues underscore the urgency in preventing exposure to C. difficile and in rapidly diagnosing and appropriately treating this disease. Adhering to these measures can dramatically improve an individual’s prognosis for recovery and limit the nosocomial spread of infection.

| ELISA | % Sensitivity<sup>a</sup> | % Specificity<sup>a</sup> | % PPV<sup>a</sup> | % NPV<sup>a</sup> |
|-------|--------------------------|------------------------|----------------|----------------|
| Premier toxin A and B | 97.44 [38/(38 + 1)] | 97.52 [393/(393 + 10)] | 79.17 [38/(38 + 10)] | 99.75 [393/(393 + 1)] |
| C. difficile TOX A/B II | 87.18 [34/(34 + 5)] | 97.27 [392/(392 + 11)] | 75.56 [34/(34 + 11)] | 98.74 [392/(392 + 5)] |

<sup>a</sup> Sensitivity = true positives/(true positives + false negatives); values shown in brackets.

<sup>b</sup> Specificity = true negatives/(true negatives + false positives); values shown in brackets.

<sup>c</sup> PPV = true positives/(true positives + false positives); values shown in brackets.

<sup>d</sup> NPV = true negatives/(true negatives + false negatives); values shown in brackets.
Studies evaluating diversity in toxin profiles among *Clostridium difficile* strains indicate that it is imperative to perform assays that evaluate specimens for the presence of both toxin A and toxin B. Further research is needed to determine the extent of iota toxin expression, the toxin’s role in causing infection, and whether an assay should be developed to evaluate its presence in clinical samples (18). Meanwhile, among the assays currently available, the traditional “gold standard” assay for toxin B is rather slow in yielding results. Similarly, among the toxin detection assays, those that test for only toxin A have serious limitations because they fail to detect toxin A-negative and toxin B-positive strains. Therefore, a highly sensitive, specific, and simple assay that allows the detection of both toxins A and B must be routinely performed.

In our study comparing the widely used Premier toxin A and B and *Clostridium difficile* TOX A/B II immunoassays, we determined that the assays performed similarly for specificity and the related NPV yet differed in sensitivity and PPV (*P* < 0.10). We attribute the association at the mere *P* of <0.10 level to the relatively low absolute number of discordant samples that remained after cytotoxin resolution. However, this notable result is strongly suggestive of highly significant sensitivity differences between the assays, and while it is outside the scope of this study, this hypothesis should be thoroughly examined.

Using blinded samples that were rotavirus-positive/ *C. difficile* toxin-negative, *C. difficile* toxin-positive, or *C. difficile* toxin-negative for evaluating test precision, we found that the two assays were comparable in intrarun results. However, the *Clostridium difficile* TOX A/B II assay has reproducibility issues, possibly due to nonspecific cross-reactivity, as it yielded false-positive results for rotavirus in two out of eight interrun comparisons (25%). We are not aware of investigations about the nature of cross-reactivity between rotavirus and toxins A and B, so cannot substantiate this aspect from a structural biology perspective.

Our preliminary experiments evaluating the detection limits of the assays' antitoxin A and antitoxin B antibodies demonstrated very similar detection limits for the antitoxin A antibodies. To further clarify the basis for the high sensitivity of the Premier toxin A and B assay compared to that of the *Clostridium difficile* TOX A/B II assay, we determined that the Premier toxin A and B polyclonal B antibody demonstrated a much lower detection limit for toxin B than the *Clostridium difficile* TOX A/B II polyclonal B antibody did for toxin B.

The *Clostridium difficile* TOX A/B II assay requires repeat evaluations of weakly positive samples (OD (1:32) = 0.200) located in wells adjacent to a high-positive sample at a rate of 2.5%. The major medical repercussions of repeat testing include the potential for misdiagnosis, failure to provide treatment in a timely manner, and failure to control the spread of infections. The obvious concern to laboratories over excessive repeat analysis is cost, related to additional personnel effort and reagent expense. Thus, the repeat analysis required by the TechLab test was unacceptable.

This study indicates that the Premier toxin A and B immunoassay is sensitive, specific, reproducible, easy to perform, and cost-effective in diagnosing *Clostridium difficile* infection. The Premier toxin A and B immunoassay detects both toxins A and B, thus reducing the probability of false-negative test results due to the presence of aberrant *C. difficile* strains that fail to produce active toxin A or B and increasing the probability of detecting all toxin A-negative/B-positive and toxin A-positive/B-negative strains. The use of this immunoassay has the potential to considerably reduce the overall medical and economic burden attributed to *Clostridium difficile* infection.

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**TABLE 5. Serially diluted toxin B binding to anti-toxin B antibodies**

| OD* | Premier toxin A and B | *Clostridium difficile* TOX A/B II |
|-----|----------------------|----------------------------------|
| 1:2 (500 pg/100 μl) | 1.324 (Pos) | 0.330 (Pos) |
| 1:4 (250 pg/100 μl) | 0.717 (Pos) | 0.160 (Pos) |
| 1:8 (125 pg/100 μl) | 0.385 (Pos) | 0.068 (Neg) |
| 1:16 (62.5 pg/100 μl) | 0.077 (Neg) | 0.035 (Neg) |
| 1:32 (31.25 pg/100 μl) | 0.028 (Neg) | 0.024 (Neg) |

*Starting concentration, 1 ng/100 μl.*

*For the Premier assay, the OD 450/630 was determined. For the TOX A/B II assay, the OD 450/620 was determined. Pos, positive test results; Neg, negative test results.*