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Is a Two-Step Glutamate Dehyrogenase Antigen-Cytotoxicity Neutralization Assay Algorithm Superior to the Premier Toxin A and B Enzyme Immunoassay for Laboratory Detection of *Clostridium difficile*?[▽]

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A two-step algorithm for the detection of *Clostridium difficile* by the use of C. Diff Quik Chek (TechLab, Blacksburg, VA) and a tissue culture cytotoxicity neutralization assay was found to be more sensitive than the widely used solid-phase enzyme immunoassay (EIA), the Premier toxin A and B EIA (Meridian Bioscience, Cincinnati, OH), and a newly developed, rapid single-test EIA for *C. difficile* toxins A and B (Tox A/B Quik Chek; TechLab).

The recent emergence of a highly virulent strain of *Clostridium difficile*, with its associated increased morbidity and increased mortality, has placed a renewed emphasis on the laboratory diagnosis of *C. difficile* disease (7, 10). For well over a decade, first the Premier toxin A enzyme immunoassay (EIA) and then the Premier toxin A and B EIA (A/B EIA; Meridian Bioscience, Inc., Cincinnati, OH) were used to detect this toxin (21). The latter is the most widely used test for the detection of *C. difficile* toxins in the United States (4). Recent studies (11, 20) have shown that this assay has both excellent sensitivity (>90%) and excellent specificity (>95%) compared to the results of a tissue culture cytotoxicity neutralization (CTN) assay, an assay frequently considered the "gold standard" for the diagnosis of *C. difficile* disease (1, 9, 11, 12, 15, 18, 19, 21). Other data suggest that the detection of toxigenic strains of *C. difficile* is actually a more sensitive approach to the diagnosis of *C. difficile* disease (5, 6, 13, 14), but culture is infrequently done for diagnostic purposes in the United States because of its slow turnaround time and the need for special isolation media and technical expertise. In addition, the specificity of toxigenic culture for the diagnosis of *C. difficile* disease has also been questioned (1, 14).

Given the emergence of a highly virulent *C. difficile* strain in the United States, a recent report by Ticehurst and colleagues was a cause for concern (18). They showed that another widely used EIA, the *C. difficile* Tox A/B II assay (TechLab, Blacksburg, VA), which has performance characteristics similar to those of the A/B EIA (11, 12, 19), had a sensitivity of only 36% for *C. difficile* detection when the results were compared to those obtained by the use of a two-step algorithm in which specimens were first tested for the presence of the glutamate dehydrogenase (GDH) antigen by a solid-phase EIA (C. Diff Chek-60; TechLab) and the positive results were confirmed by the CTN assay. The test for the GDH antigen proved to be an excellent screening test, with a sensitivity of >95% and a negative predictive value of >99% compared with the results of the CTN assay (18). In practical terms, that meant that GDH-negative specimens could be reported to be negative without further testing. By using the CTN assay as a reference method, it was found that the assay for the GDH antigen had a positive predictive value of 53%, indicating that a confirmatory test was required to detect specimens containing *C. difficile* toxins.

I applied the two-step algorithm approach using a modification of the GDH antigen assay, which used a recently developed membrane-bound lateral flow device, C. Diff Quik Chek (TechLab), and compared its performance characteristics to those of the A/B EIA with a membrane-bound lateral flow device (Tox A/B Quik Chek; TechLab) for the detection of toxins A and B (the LF-A/B assay).

Three hundred sixty-eight clinical specimens submitted between April and June 2007 for the detection of *C. difficile* toxin by the A/B EIA were studied. The inclusion criteria were as follows: a clinician had to order the detection of *C. difficile* toxin in a specimen, the specimen had to be less than 24 h old, the specimen had to take the form of the specimen container, and the specimen volume had to be sufficient to allow performance of the four assays that were used in this study. All specimens were stored at 4°C prior to assay. The A/B EIA, the C. Diff Quik Chek assay (the GDH assay), the LF-A/B assay, and the CTN assay with the TechLab *C. difficile* toxin B assay were all done according to the manufacturer's instructions, with the exception that the results for specimens with optical densities at 450/630 nm of 0.1 to 0.5 by the A/B EIA were confirmed by the CTN assay. In-house data have shown that only 30% of specimens with optical density values between 0.1 and 0.5 by the A/B EIA could be confirmed to be positive by the CTN assay. Positive controls were run on each day of the assay for all tests. In addition, a negative control was run for the A/B EIA, while each of the GDH and LF-A/B assays has

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TABLE 1. Performance characteristics of A/B EIA and the LF-A/B assay compared with those of a two-step algorithm using C. Diff Chek-60 and *C. difficile* toxin B assays

Test and result	No. of specimens with the following two-step algorithm result:		Sensitivity (%)	Specificity (%)	NPV[a] (%)	PPV[b] (%)
	Positive	Negative				
A/B-EIA			59.5 (43.5–73.7)[c]	99.4 (97.8–99.8)	95.6	91.7
Positive	22	2				
Negative	15	329				
LF-A/B assay			43.2 (28.7–59.1)	98.5 (96.5–99.4)	93.9	76.2
Positive	16	5				
Negative	21	326				

[a] NPV, negative predictive value (predictive value of a negative test result).
[b] PPV, positive predictive value (predictive value of a positive test result).
[c] Values in parentheses are the 95% confidence limits, determined by the "score" method.

an internal negative control. The individual performing the GDH and the LF-A/B assays was blinded to the A/B EIA results.

For the purposes of comparisons of the algorithm with the A/B EIA and the LF-A/B assays, specimens were assigned a true-positive result by the two-step algorithm if they were both GDH assay positive and CTN assay positive; they were assigned a true-negative result if they GDH assay negative or GDH assay positive but CTN assay negative. These designations are based on the high sensitivity and the high negative predictive value previously reported for this algorithm (18). Confidence intervals were determined by the use of EP Evaluator software (David G. Rhoads Associates, Kennett Square, PA). This study was approved by the Institutional Review Board of the University of North Carolina.

Of the 368 specimens studied, 70 were positive and 298 were negative by the GDH assay.

Of the 70 GDH assay-positive specimens, 37 were confirmed to contain *C. difficile* toxin by the CTN assay. Only 22 of the 37 specimens confirmed to have positive results were positive by the A/B EIA, resulting in an A/B EIA sensitivity of 59.5%. The sensitivity was even lower for the LF-A/B assay (43%). The positive predictive values, the negative predictive values, and the specificities of the EIAs were all very good to excellent (Table 1). Two specimens were found to be GDH assay negative but A/B EIA positive. Both specimens were negative by the CTN assay. The results for five specimens found to be positive by the LF-A/B assay could not be confirmed by the CTN assay; two of the five specimens were also positive by the GDH assay.

The use of two-step diagnostic algorithms, in which an easily performed and highly sensitive but less specific assay is used as a screening test to eliminate specimens with negative results and in which the results for specimens with positive results are confirmed by a more specific but often more complex confirmatory test, is a well-established diagnostic paradigm. Such algorithms are widely used for the diagnosis of both syphilis and human immunodeficiency virus infection (3). In the present study, I wanted to learn if the two-step algorithm described by Ticehurst et al. (18) would enhance the ability to detect *C. difficile* toxin compared to the ability of the most commonly used *C. difficile* toxin A and B EIA, the A/B EIA (Meridian Bioscience). Although the differential was not as dramatic as that reported by Ticehurst et al. (18), I found that the two-step algorithm had an enhanced ability to detect *C. difficile* toxin-positive specimens by 40% compared to the results of the A/B EIA. I also evaluated a novel lateral flow toxin A and B EIA, the LF-A/B assay, but found it to be both less sensitive and less specific than the A/B EIA.

What are some of the practical observations made during this study? First, the LF-A/B assay had performance characteristics very similar to those reported for the C. Diff Chek-60 assay (16, 18) in that it had a positive predictive value of only 53% but a sensitivity of 100% compared to tissue culture results. Because the results for all negative specimens were not confirmed by the CTN assay, the specificity and the negative predictive value of this GDH assay cannot be stated with certainty. However, 293 of 298 of the GDH assay-negative specimens were also negative by the two other immunoassays that were studied, with the A/B EIA having been shown to be highly specific and to have a high negative predictive value in several studies (9, 11, 12, 15, 19). The seven GDH assay-negative specimens that were positive by one of the two other EIAs were found to be negative by the CTN assay. These data suggest that the LF-A/B assay does have a very high negative predictive value, a crucial characteristic of any screening test, but because of the study design, this cannot be proven conclusively.

Second, the GDH screening test is easily performed and the results are easily interpreted. The test takes approximately 20 min to perform and is very simple, and the filters clogged for less than 5 of 368 specimens tested. There was one failed run during the study because the reagents were not added in the proper order. This was determined by failure of the internal control. I used positive controls on each day of use. Except for the failed run, the positive control gave an appropriate reaction each time, suggesting that positive controls need to be tested only once per test lot. My laboratory can currently offer this testing three times per day, and stat testing is also easily accomplished. My laboratory can offer testing by the A/B EIA only once a day, and stat testing is inconvenient and is rarely done.

Third, this approach is more expensive, and for an estimated 20% of specimens that require the CTN assay, the turnaround time is delayed compared to the turnaround time for the EIA. The material costs per specimen for the A/B EIA were approx-

imately $5.00, while the current per specimen cost for the LF-A/B assay is $9.00. In addition, 20% of specimens require the CTN assay, which has an approximate cost of $8.00. These specimens require both centrifugation and filtration, which are labor-intensive and which thus add further to the cost of this algorithm. The overall material cost per specimen for the two-step algorithm is $10.60. In order to provide the best possible turnaround time, testing by the CTN assay must be offered 7 days/week.

A major question concerning this study is the explanation for the discrepancy between the high sensitivities (>90%) for the A/B EIA reported recently (11, 20) and the comparatively modest sensitivity found in this study (59.5%). It is possible that there were systematic errors in the performance of this assay during the study period. However, quality control failures have been rare, and the lack of proficiency test failures since the institution of this assay in my laboratory makes this type of error less likely. Alternatively, there may have been a regional shift in the antigenic nature of the toxin, resulting in the EIA becoming less sensitive. I did notice that several of the GDH assay- and CTN assay-positive but A/B EIA-negative specimens were CTN positive only after 48 h of incubation, suggesting the presence of low levels of toxin and perhaps explaining the inability of the EIA to detect the toxin. However, a similar number of EIA-negative specimens were GDH and CTN assay positive at 24 h, suggesting that a low toxin level was not the entire explanation for the relative insensitivity of the A/B EIA.

The data presented here and by others (2, 6, 9, 16, 18) indicate that GDH detection is the most sensitive and convenient method by which *C. difficile* can be detected in stools. The assay also has a high negative predictive value. Because of this test's modest positive predictive value of approximately 50%, a rapid, convenient confirmatory test for GDH-positive specimens is needed before this test is likely to gain wide acceptance in clinical laboratories. The data presented here and by others (18) indicate that two solid-phase EIAs, the *C. difficile* Tox A/B II EIA (TechLab) and the A/B EIA (Meridian Bioscience), as well as a single-test lateral flow immunochromatographic test, the LF-A/B assay (TechLab), lack sufficient sensitivity compared with the results of the CTN assay to be used as confirmatory tests. For laboratories wishing to implement screening for *C. difficile* by the GDH assay as part of a testing algorithm, for now it is necessary to use either the CTN assay or toxigenic culture (5, 6, 14) as a confirmatory test for GDH assay-positive specimens.

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