Clostridium difficile Testing in the Clinical Laboratory by Use of Multiple Testing Algorithms

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The incidence of Clostridium difficile infection (CDI) has risen almost 3-fold in the United States over the past decade, emphasizing the need for rapid and accurate tests for CDI. The Cepheid Xpert C. difficile assay is an integrated, closed, nucleic acid amplification system that automates sample preparation and real-time PCR detection of the toxin B gene (tcdB). A total of 432 stool specimens from symptomatic patients were tested by a glutamate dehydrogenase (GDH) assay, a toxin A and B enzyme immunoassay (EIA), the Xpert C. difficile assay, and a cell culture cytotoxicity neutralization assay (CCCN). The results of these methods, used individually and in combination, were compared to those of toxigenic culture. Results for the Xpert C. difficile assay alone showed a sensitivity, specificity, positive predictive value, and negative predictive value (NPV) of 94.4, 96.3, 84.0, and 98.8%, while the EIA alone gave corresponding values of 58.3, 94.7, 68.9, and 91.9%, respectively. An algorithm using the GDH assay and the EIA (plus the CCCN if the EIA was negative) showed corresponding values of 83.1, 96.7, 83.1, and 96.1%. The Xpert C. difficile assay was statistically superior to the EIA (P <0.001 by Fisher's exact test) and to the GDH-EIA-CCCN algorithm (P = 0.0363). Combining the GDH and Xpert C. difficile assays lowered both the sensitivity and the NPV of the Xpert assay. The GDH-EIA-CCCN procedure required, on average, 2 days to complete testing on GDH-positive results, while testing by the Xpert C. difficile assay was completed, on average, in less than 1 h. Xpert C. difficile testing yielded the highest sensitivity and NPV, in the least amount of time, of the individual- and multiple-test algorithms evaluated in this study.

Clostridium difficile is the main cause of infectious health care-associated diarrhea in the United States and around the world. C. difficile infections (CDI) can vary from a mild diarrhea to the potentially fatal pseudomembranous colitis, toxic megacolon, and sepsis (7, 19). C. difficile colonization of the bowel often follows disruption of normal flora after the patient receives antimicrobial therapy. The incidence and severity of C. difficile in both hospital and long-term care settings, due in part to the emergence of several novel strains, including the epidemic J strain described by Johnson et al. and the hypervirulent NAP1/027/BI strain (16, 19). Strain NAP1/027/BI produces high quantities of spores, which disseminate easily in the hospital environment, and is associated with high mortality rates (1, 5, 9, 11, 17, 32).

Historically, the cell culture cytotoxicity neutralization assay (CCCN), which detects cytotoxin production in monolayers of cells, such as human diploid fibroblasts, has been the gold standard for C. difficile detection in the laboratory. However, cell culture is labor-intensive, and many laboratories have adopted other testing methods, such as enzyme immunoassays (EIAs) for toxins A and B, which are easier and faster to perform than CCCN (22, 27, 33). However, recent reports have highlighted the lack of sensitivity of the toxin A/B EIAs, which show sensitivities as low as 48% (2, 28). Although toxigenic culture of the organism has now been accepted as the true gold standard (25), this method requires substantial laboratory resources, and results are not available in a short enough time frame to be clinically useful (18, 24, 28). Thus, other approaches to improving both the sensitivity and the cost-effectiveness of C. difficile testing have been introduced (2, 22). Testing algorithms using a glutamate dehydrogenase (GDH) assay (which has presumptively higher sensitivity but lacks specificity) to screen for C. difficile in stool samples, with reflex testing using a more specific assay, such as a toxin A/B EIA or the CCCN, have been proposed (26, 29, 31). GDH assays detect antigen present in both toxigenic and nontoxigenic strains of C. difficile directly in stool samples. The time necessary to perform the GDH assay with EIA or CCCN confirmation can be as long as 3 days (34). Gilligan noted that EIAs often lack sufficient sensitivity for confirmation of positive GDH assay results (14). In this algorithm, the need to confirm GDH-positive specimens increases the turnaround time (TAT) for positive results, delaying the notification of the physician ordering the test. PCR assays for various targets have been developed as a potential replacement for the less-sensitive (EIA) and less-specific (GDH) assays for C. difficile detection (3, 4, 6, 23, 30). Such assays include both “home brew” PCR assays and FDA-cleared commercial assays (15, 20, 28, 30). Cepheid (Sunnyvale, CA) has recently developed a GeneXpert cartridge-based assay for detection of the C. difficile toxin B gene (tcdB) directly from stool. In this study, we compared the sensitivity and specificity of the Xpert C. difficile PCR assay to...
those of the GDH assay and the EIA, individually and within specific testing algorithms, using toxigenic culture as the gold standard for a positive specimen. (This work was previously presented as a poster at the 109th General Meeting of the American Society for Microbiology, 2009.)

MATERIALS AND METHODS

Study population and sample collection. This was a prospective study conducted at the Southern California Permanente Medical Group Regional Reference Laboratories. Eligible patients included those with suspected CDI for whom toxin EIAs from unformed stool were ordered for C. difficile testing according to the institution’s standard practices. All specimens enrolled and tested in this study represented excess, leftover stool, and therefore, informed consent was waived by the Institutional Review Board. Duplicate specimens from the same patient and patients under the age of 2 years were excluded.

Upon the establishment of eligibility and following standard testing at the institution, excess unformed stool specimen was placed in a sterile container. A portion of the specimen was shipped to a single central laboratory for reference culture. The remaining unformed stool was used for testing by the EIA, the GDH assay, and the Xpert C. difficile assay on site. Those specimens that were negative for C. difficile by the EIA but positive by the GDH assay were further tested for cytotoxicity B by the CCCN.

Enzyme immunosassay. Toxin testing was performed using the Premier Toxins A & B microwell EIA (Meridian Bioscience, Inc., Cincinnati, OH). The assay was performed according to the manufacturer’s instructions. Positive results for the Premier Toxins A & B EIA are indicated by optical densities at 450 and 630 nm (OD450/630) of $>0.10$ and negative results are indicated by OD450/630 of $<0.10$.

Glutamate dehydrogenase. Specimens were tested for GDH using the TechLab C. DIFF CHEK-60 EIA (distributed by Inverness Medical Innovations, Inc., Princeton, NJ). Testing was performed on previously frozen stool specimens according to the manufacturer’s instructions. Negative results for GDH antigen are indicated by OD450/630 of $<0.08$ and positive results are indicated by OD450/630 of $>0.08$. Specimens that were positive for GDH were tested by EIA. GDH-positive, EIA-negative specimens were considered positive for toxin B-producing C. difficile. Those specimens that were GDH positive and EIA-negative were tested by the CCCN. GDH-positive, EIA-negative, CCCN-positive specimens were considered positive for toxin B-producing C. difficile.

Xpert C. difficile PCR assay. The Xpert C. difficile PCR assay (Cepheid, Sunnyvale, CA) was performed according to the manufacturer’s instructions. Briefly, a swab was dipped into the unformed stool specimen container. The swab was placed in sample reagent and capped. The specimen was vortexed for 10 s, and all the liquid from the sample reagent was transferred to the “S” chamber of the instrument according to the manufacturer’s instructions. Positive results for the Xpert Toxins A & B EIA are indicated by optical densities at 450 and 630 nm (OD450/630) of $>0.10$ and negative results are indicated by OD450/630 of $<0.10$.

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Clinical performance. The performances of the GDH assay, EIA, and Xpert C. difficile assay as individual indicators and as parts of testing algorithms for the presence of toxin B-producing C. difficile were assessed for 432 stool samples; the results of each assay were compared to the results of toxigenic culture. In this study, both direct toxigenic and enriched toxigenic cultures were performed. If the direct toxigenic culture was negative, then the results of the enriched toxigenic culture were evaluated. Four specimens failed to yield a valid PCR result by use of the Xpert C. difficile assay (n = 428). For the GDH-EIA-CCCN algorithm, one cytotoxicity specimen gave no result, due to bacterial contamination (n = 431). Only four samples yielded nontoxicigenic C. difficile isolates. Relative to toxigenic culture, the Xpert C. difficile assay demonstrated a sensitivity, a specificity, and positive and negative predictive values of 94.4, 96.3, 94.0, and 98.8%, respectively, while the EIA demonstrated a sensitivity, a specificity, and positive and negative predictive values of 58.3, 94.7, 68.9, and 91.9%, respectively (Table 1).

Compared to toxigenic culture, the algorithm of the GDH assay and the EIA yielded a sensitivity, a specificity, and positive and negative predictive values of 55.6, 98.3, 87.0, and 91.7%, respectively, and the algorithm of the GDH assay combined with the toxin EIA and the CCCN (if the confirmation EIA gave a negative result) demonstrated values of 83.1, 96.7, 83.1, and 96.7%, respectively (Table 1). The GDH assay com-

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TABLE 1. Summary of algorithm versus stand-alone testing options compared to direct/enriched toxigenic culture

<table>
<thead>
<tr>
<th>Parameter</th>
<th>EIA only</th>
<th>GDH + EIA</th>
<th>GDH + EIA + cytotoxin&lt;sup&gt;a&lt;/sup&gt;</th>
<th>GDH + Xpert&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Xpert only&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of specimens</td>
<td>432</td>
<td>432</td>
<td>431</td>
<td>432</td>
<td>428</td>
</tr>
<tr>
<td>Sensitivity Pos</td>
<td>58.3 (42/72)</td>
<td>55.6 (40/72)</td>
<td>83.1 (59/71)</td>
<td>86.1 (62/72)</td>
<td>94.4% (68/72)</td>
</tr>
<tr>
<td>Specificity</td>
<td>94.7 (341/360)</td>
<td>98.3 (354/360)</td>
<td>96.7 (348/360)</td>
<td>97.8 (352/360)</td>
<td>96.3 (343/356)</td>
</tr>
<tr>
<td>Accuracy</td>
<td>88.7 (383/432)</td>
<td>91.2 (394/432)</td>
<td>94.4 (407/431)</td>
<td>95.8 (414/432)</td>
<td>96.0 (411/428)</td>
</tr>
<tr>
<td>PPV</td>
<td>68.9 (42/61)</td>
<td>87.0 (40/46)</td>
<td>83.1 (59/71)</td>
<td>88.6 (62/70)</td>
<td>84.0 (68/81)</td>
</tr>
<tr>
<td>NPV</td>
<td>91.9 (341/371)</td>
<td>91.7 (354/386)</td>
<td>96.7 (348/360)</td>
<td>97.2 (352/362)</td>
<td>98.8 (343/347)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV) are expressed as percentages followed by fractions in parentheses as follows: sensitivity, (number of true-positive results)/(sum of true-positive and false-negative results); specificity, (number of true-negative results)/(sum of true-negative and false-positive results); accuracy, (number of true results)/(total results); PPV, (number of true-positive results)/(sum of true-positive and false-positive results); NPV, (number of true-negative results)/(sum of true-negative and false-negative results).

<sup>b</sup> Cytotoxin assay results were not available for one specimen.

<sup>c</sup> The four specimens with no Xpert results were GDH negative, so according to the algorithm, no Xpert test was needed.

<sup>d</sup> Xpert results were not available for four specimens.

bined with the Xpert C. difficile PCR assay demonstrated both lower sensitivity (86.1%) and a lower negative predictive value (97.8%) than the Xpert C. difficile assay alone (Table 1). The addition of the GDH screening test increased the turnaround time for most results. The GDH-EIA-CCCN results were available, on average, in 48 h, while the results of the Xpert C. difficile assay were available, on average, in less than 1 h.

Of the 13 patient specimens that were positive by the Xpert C. difficile PCR assay and negative by toxigenic culture, three specimens were positive by the GDH assay, the EIA, and the CCCN; two specimens were GDH positive, EIA positive, and CCCN negative; two specimens were GDH positive, EIA negative, and CCCN positive; one specimen was GDH positive, EIA negative, and CCCN negative; three specimens were GDH negative, EIA negative, and CCCN negative; and two specimens were negative by the GDH assay, the EIA, and the CCCN (Table 2). Of the four specimens that were negative by the Xpert C. difficile assay but culture positive, three were GDH positive, EIA negative, and CCCN negative (Table 2). Eleven of the 17 specimens for which discrepant results were obtained came from patients with histories of C. difficile infection.

The results of a categorical analysis of the data across the different methods showed that the methods gave statistically different results for sensitivity (P \( \leq 0.0001 \) \( [P < 0.0001 \) both by Fisher’s exact test and by the chi-square test]). However, the P value for specificity was \( > 0.05 \) (P = 0.0657 by Fisher’s exact test and 0.0603 by the chi-square test), indicating that the different methods are statistically equivalent for specificity, although the lower specificity of the EIA approached significance (data not shown). There was no statistical difference between the sensitivity result for the Xpert (58.3%) and that for the combination of the GDH assay and the toxin EIA (55.6%) (P = 0.8664 by Fisher’s exact test) or between the sensitivity result for the Xpert C. difficile assay (94.4%) and that for the GDH assay–Xpert C. difficile assay algorithm (86.1%) (P = 0.1578), which was surprising given the large gap between the sensitivities, although the lack of significant difference may have been due to the relatively small number of positive samples in this study. There were, however, statistically significant differences between the sensitivity result for the Xpert C. difficile assay alone and that for the toxin EIA alone (P < 0.001) and between the sensitivity result for the Xpert C. difficile assay alone and that for the GDH-EIA-CCCN three-test algorithm (P = 0.0363).

DISCUSSION

CDI is a major medical and infection control problem in many health care facilities, including hospitals, long-term care facilities, and nursing homes around the world (8, 20). Accurate and timely diagnosis is necessary both for appropriate clinical management of the patient and for the timely implementation of infection control and pharmacy measures (13, 21). Many hospitals are now required to report health care-associated transmission of pathogens, including C. difficile, to public health departments. Thus, it is imperative that the diagnosis of CDI be rapid and accurate.

Due to the poor sensitivity of the toxin A/B EIA results who are still suspected of having CDI are often subjected to additional testing and procedures, adding substantial costs to hospitalization (14, 19). Per-
haps more importantly, a significant number of active *C. difficile* carriers are undiagnosed, leading to unmitigated transmission in health care settings. Often one or more stool specimens are collected following the first EIA-negative result if the suspicion of CDI is high (10, 20). Algorithms for *C. difficile* testing in the hospital setting have evolved due to the need for more-accurate assays to better detect those patients with CDI (26, 31). Algorithms that appear to be more sensitive than testing by the toxin A/B EIA alone have been implemented in laboratories (26, 29, 31). These algorithms use the GDH assay as a screen and confirm GDH-positive results with the toxin A/B EIA, or with either culture or the CCCN. In previously published data, the GDH assay appeared to have good sensitivity in detecting *C. difficile*-positive specimens but lacked specificity (29). Two recent studies evaluated a multistep approach with the GDH assay as a screening assay and found that as many as 23 to 24% of the positive specimens were missed by using this algorithm (i.e., sensitivity was approximately 76%) (26, 28). In our study, using a multistep approach, we achieved a sensitivity of 83.1% compared to toxigenic culture by using the GDH assay as the initial screen. These results are slightly lower than those reported by Eastwood et al. (12), who noted a GDH assay sensitivity of 87.6% compared to toxigenic culture in their study.

Although these multistest algorithms improve the specificity of the GDH test, they delay the reporting of the results to the ordering physician (14). Multitest algorithms often require 2 or more days to validate a positive screening result, particularly if the CCCN is used for confirmation. Although many physicians start empirical therapy when a patient is suspected of having CDI, others wait for the results of the diagnostic tests to confirm the diagnosis of CDI before stopping other antibiotics and initiating specific CDI therapy. This delay, especially in combination with the low sensitivity of the EIA, can facilitate the spread of *C. difficile* to other patients and further contamination of the environment, in addition to increasing the potential for the patient to develop more-severe disease (21).

Our data demonstrate that the sensitivity and negative predictive value of the Xpert *C. difficile* assay exceed those of the other individual assays and testing algorithms, specifically the GDH-EIA-CCCN algorithm (P = 0.0363), which is superior to the GDH-EIA algorithm in terms of sensitivity (83.1% versus 55.6%, respectively). This study confirms other published data demonstrating that the sensitivity of the EIAs currently marketed is very limited; thus, they should not be considered reliable for the detection of CDI. A review of the other testing results for specimens that were positive by the Xpert *C. difficile* assay but negative by reference toxigenic culture shows that 7 of 13 specimens appear to be true-positive specimens, given the positive GDH, EIA, and/or CCCN results (Table 2). The corrected sensitivity and specificity of the PCR assay would be 95.1% and 99.4%, respectively. Negative results by reference toxigenic culture could be due to several factors, such as patients’ receipt of antimicrobial agents at the time of specimen collection, the lag time between specimen collection and testing, or the presence of nonviable organisms in the sample. Lack of either heat or alcohol shock for spore enrichment in the culture protocol may also have contributed to these seemingly false-negative culture results. Conversely, three of the four specimens that were negative by the Xpert *C. difficile* assay but positive by reference toxigenic culture were positive by the GDH assay but both EIA negative and CCCN negative (Table 2), which may be explained by a sampling problem due to low concentrations of organisms in very heterogeneous samples. Taken together, however, these data suggest that the sensitivity and specificity of the Xpert *C. difficile* assay would allow the laboratory not only to enhance and speed up the detection of toxigenic *C. difficile* beyond that with EIAs and GDH assay-based algorithms but also to have even more confidence in an initially negative result based on one test only. Further studies will be needed to determine the significance of PCR-positive results for patients who test negative by other conventional methods.

Two other PCR assays for *C. difficile* have recently been cleared by the FDA for use in U.S. laboratories. The results of those FDA-cleared assays were compared to the results of the CCCN rather than to the results of toxigenic culture in the clinical trials used for FDA clearance (30). Compared to the CCCN, these assays performed well. However, Stamper et al. compared the GeneOhm assay from Becton Dickinson & Company (BD) to toxigenic culture and demonstrated a sensitivity of 83.6%, which was lower than stated in the package insert for the BD product (30). Eastwood et al. (12) reported a somewhat higher sensitivity (88.5%) for the BD assay compared to toxigenic culture. Gluck et al. (15) compared the Prodesse proGASTRO *C. difficile* PCR assay to toxigenic culture and reported a sensitivity of 73.3% and a specificity of 99.2%. Since toxigenic culture has better sensitivity than the CCCN alone for detecting *C. difficile*, it is not surprising that the sensitivities reported by Stamper et al. and Gluck et al. when comparing the assays to the gold standard are lower than those reported in the package inserts (30). The sensitivity of the Xpert *C. difficile* assay was determined in this study by comparing the results to those of enriched toxigenic culture. A study comparing the results of all three commercial PCR methods for the same stool specimen to those of enriched toxigenic culture would be of interest.

The cost-benefit ratio of the PCR-based assays, which will be more expensive than the GDH-EIA-CCCN algorithm, needs to be determined. Due to the improved sensitivity that PCR assays provide, theoretically, physicians would be able to order fewer *C. difficile* tests in order to obtain an accurate diagnosis (25). This potentially offsets some of the increased cost of the newer PCR assays. Hospitals need to consider what TAT is needed for *C. difficile* results, as well as the impact of TAT on the risk of transmission. Patient outcome should be considered in addition to other data in determining what approach to *C. difficile* testing is required in the laboratory setting. We conclude that when the various algorithms are compared, the Xpert *C. difficile* assay, both with regard to sensitivity and with regard to the negative predictive value, exceeds the performance levels of the individual tests and the GDH-EIA-CCCN algorithm and may also be able to reduce the practice of multiple test ordering, which has become commonplace in many hospitals.

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REFERENCES


