Clostridium difficile Testing Algorithms Using Glutamate Dehydrogenase Antigen and C. difficile Toxin Enzyme Immunoassays with C. difficile Nucleic Acid Amplification Testing

Increase Diagnostic Yield in a Tertiary Pediatric Population

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Running Title: C. difficile diagnosis in children

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Abstract

We evaluated the performance of the rapid C. DIFF QUIK CHEK COMPLETE’s glutamate dehydrogenase antigen (GDH) and toxin A/B (CDT) tests in two algorithmic approaches in a tertiary pediatric population: algorithm #1 - initial testing with GDH/CDT followed by Loop-Mediated Isothermal Amplification (LAMP) and algorithm #2 - GDH/CDT followed by cytotoxicity neutralization assay (CCNA) for adjudication of discrepant GDH-positive/CDT-negative results. True positive (TP) was defined as positivity by CCNA or positivity by LAMP plus another test (GDH, CDT or the Premier C. difficile Toxin A & B enzyme immunoassay (P-EIA)). 141 specimens from 141 patients yielded 27 TPs and 19% prevalence. Sensitivity, specificity, positive predictive value and negative predictive value were 56%, 100%, 100%, 90% for P-EIA and 81%, 100%, 100% and 96% for both algorithm #1 and algorithm #2. In summary, GDH-based algorithms detected C. difficile infections with superior sensitivity compared to P-EIA. The algorithms allowed immediate reporting of half of all TPs but LAMP or CCNA was required to confirm the presence or absence of toxigenic C. difficile in GDH+/CDT- specimens.
Introduction

*Clostridium difficile* infection (CDI) is a leading cause of antibiotic-associated colitis in children and is associated with significant morbidity in vulnerable pediatric populations (4, 11). The optimal testing method for laboratory detection of CDI remains controversial in both adults and in children (18). Suboptimal sensitivity and specificity of many commercial enzyme immunoassays has limited their utility to the extent that the 2010 IDSA-SHEA Clinical Practice Guidelines for *Clostridium difficile* Infection in Adults discouraged their use (2, 3). Cell culture cytotoxicity neutralization assay (CCNA), the traditional gold standard, performs with superior sensitivity but is labor intensive and requires 48 hours to finalize. Nucleic acid amplification tests (NAATs) including BD GeneOhm Cdiff (BD Diagnostics, San Diego, CA), Prodesse ProGastro Cd (Gen-Probe Inc, San Diego, CA), Xpert C.difficile (Cepheid, Sunnyvale, CA) and *illumi*gene C. difficile (Meridian Biosciences, Cincinnati, OH) appear to perform with sensitivity equaling or exceeding that of CCNA.

In the adult literature, multi-step diagnostic algorithms have been evaluated in an effort to use NAATs more judiciously (1). The most recent iteration of this approach involves initial testing of stool specimens submitted for *C. difficile* toxin testing with the rapid *C. DIFF QUIK CHEK COMPLETE* assay (10, 12, 15). This product has two enzyme immunoassay components that detect glutamate dehydrogenase antigen (GDH) and toxin A/toxin B (CDT) in a lateral flow device. Typically, specimens that test positive or negative by both GDH and CDT are reported immediately as “positive” or “negative” for *C. difficile* toxin respectively. Because this assay detects the presence of both toxigenic and non-toxigenic *C.difficile* strains, GDH-positive/CDT-negative specimens require additional testing to adjudicate GDH+/CDT- specimens. Data in the literature suggest that algorithmic approaches perform with superior sensitivity and specificity.
compared to commercial enzyme immunoassays, with sensitivity and specificity ranging from 98-100% and 88-97% respectively (10, 12, 15). The negative predictive value (NPV) of GDH and the positive predictive value (PPV) of GDH-positive/CDT-positive results have been reported to be close to 100% (10, 12, 15), supporting the practice of immediately reporting these results without further testing. The high NPV of GDH also obviates the need to pursue the controversial practice of repeat testing of negative patients (1, 2). Finally, while some centers that process large volumes of *C. difficile* toxin tests may find performance of molecular testing routinely on all submitted specimens cost effective (compared to use of two tests), an algorithmic approach may be more cost effective in others (1, 18).

To our knowledge, algorithmic approaches to CDI detection have not been evaluated in children. This study evaluated the performance of two algorithms for detection of CDI in a tertiary pediatric population: 1. Initial testing with GDH/CDT followed by LAMP to resolve discrepant GDH-positive/CDT-negative specimens (algorithm #1) and 2. Initial testing with GDH/CDT followed by CCNA to resolve discrepant specimens (algorithm #2).

**Materials and Methods**

**Patients and specimens**

This was a prospective observational study. Consecutive, fresh, liquid or non-formed stool specimens collected from patients 1-18 years of age and submitted to the microbiology laboratory for *C. difficile* toxin testing at The Children’s Hospital of Philadelphia between March 1 and March 30, 2011 were included. Specimens were excluded from analysis if the patient had been previously tested by the study protocol.

All specimens were tested prospectively using the *C. DIFF QUIK CHEK COMPLETE* (GDH/CDT; Techlab, Blacksburg, VA); the Premier *C. difficile* Toxin A & B enzyme
immunoassay (P-EIA; Meridian Biosciences, Cincinnati, OH), cell cytotoxicity neutralization assay (CCNA; Tox-B Test; Techlab, Blacksburg, VA) and Loop-Mediated Isothermal Amplification (LAMP) using the illumigene C. difficile assay (Meridian Biosciences, Cincinnati, OH). All tests were performed according to the instructions and within the timeframes recommended by the assay manufacturers. Specimens were stored at 2-8°C while awaiting testing. External positive and negative controls were performed for each assay with each new lot and each time the assay was used.

GDH/CDT results were interpreted as follows. The test device was examined for the appearance of blue dots in the middle of the reaction window, representing the internal positive control. The test result was interpreted as invalid if the internal control dots were not present. The device was then examined for the appearance of blue lines on the “Ag” and “Tox” sides of the reaction window. The appearance of a blue line on the “Ag” side was read as “GDH-positive”. The appearance of blue lines on both the “Ag” and “Tox” sides was interpreted as “GDH-positive” and “CDT-positive”.

CCNA was performed using the C. difficile Tox-B Test kit. Briefly, following dilution, stool was centrifuged and the supernatant filtered using a 0.45 µm syringe filter. The stool filtrate was then inoculated on to a human foreskin fibroblast cell line (Diagnostic Hybrids, Athens, OH) with and without C. difficile anti-toxin. The cell line was incubated at 37°C in 5-10% CO₂ for 48 hours. Light microscopy was used to read the cell line plates at 24 and 48 hours. Cytotoxic activity was considered positive if ≥50% of the cells in a well were rounded. Stool filtrates that produced cell cytopathic effect that was inhibited by C. difficile anti-toxin were considered “positive” for C. difficile toxin. Specimens were tested by CCNA within 24 hours of collection.
LAMP is a nucleic acid amplification test that targets a 204 bp region of the \textit{tcdA} gene that resides within the pathogenicity locus of toxigenic \textit{C. difficile}. It was performed according to manufacturer’s instructions for the \textit{illuminigene C. difficile} assay. At the conclusion of the run, results were reported as “Positive”, “Negative” or “Invalid”.

Patient charts were reviewed retrospectively to understand the clinical relevance of specimens that tested LAMP-positive/CCNA-negative and CCNA-positive/LAMP-negative. This component of the study received approval by the institutional review board at The Children’s Hospital of Philadelphia.

**Statistical analysis**

A composite gold standard was initially used for analysis. A specimen was considered “true positive” for \textit{C. difficile} toxin if it was positive by CCNA. If CCNA was negative, the specimen could also be considered “true positive” if the specimen tested positive by LAMP and by another test (GDH, CDT or P-EIA). For the purpose of analysis, specimens were categorized as “true negative” if they did not meet criteria as a “true positive”. Prevalence, sensitivity, specificity, PPV and NPV were calculated for GDH, CDT, P-EIA, CCNA, LAMP, and two algorithms – GDH/CDT followed by LAMP to resolve discrepant GDH-positive/CDT-negative specimens (algorithm #1) and GDH/CDT followed by CCNA to resolve discrepant specimens (algorithm #2). The McNemar test was used to compare the sensitivity of each algorithm with P-EIA, our current testing method. The analysis was then repeated using CCNA positivity as the sole gold standard criterion.

**Results**

141 stool specimens meeting inclusion criteria were collected and tested from 141 consecutive patients during the study period. Testing was complete for all 141 specimens. The
mean and median time interval from specimen collection to testing were 17.1 and 18.2 hours for GDH/CDT; 23.0 and 19.6 hours for P-EIA, 16.7 and 17.7 hours for CCNA, 49.3 and 49.5 hours for LAMP. The sensitivity, specificity, PPV and NPV of GDH, CDT, P-EIA, CCNA, LAMP, algorithms #1 and #2 using the composite definition of “true positive” and the CCNA definition of “true positive” are summarized in Table 1.

Using the composite definition of “true positive”, twenty-seven (27) specimens were “true positive” for *C. difficile* toxin, resulting in a prevalence of 19%. Of these, 26 met the definition through a positive CCNA result. The remaining specimen was positive by LAMP and GDH. GDH was positive in 42 specimens, performing with a sensitivity of 81% and specificity of 82%. PPV was 52% and NPV was 95%. The CDT component detected 13 true positives with a sensitivity of 48% and specificity of 100%. All specimens that were positive by CDT were also positive by GDH, CCNA and LAMP. P-EIA was positive in 15 specimens with a sensitivity of 56% and specificity of 100%. PPV and NPV were 100% and 90% respectively. CCNA detected 26 positives with sensitivity of 96% and specificity of 100%. LAMP detected 24 positives with a sensitivity of 89% and specificity of 98%. The sensitivities of both GDH-based algorithms were significantly higher than that of P-EIA by McNemar testing (p<0.001). Using CCNA as the sole gold standard criterion, sensitivity, specificity, PPV and NPV were all within three percentage points of those calculated using the composite gold standard definition.

With respect to discordance, three specimens were CCNA-positive/LAMP-negative. All three were GDH and P-EIA-negative. There were also three LAMP-positive/CCNA-negative specimens. One was GDH-positive but the the remaining two specimens were negative by all other tests. CCNA and LAMP were repeated on all six discordant specimens and yielded identical test results. Chart review of the discordant cases revealed that all six cases had diarrhea.
that met the definition provided by the 2010 SHEA/IDSA guidelines (3 diarrheal stools or
greater within 24 hours or less) (2). None of the six cases had any other documented positive C.
difficile toxin test results. Review of the LAMP-positive/CCNA-negative cases suggested false
positive LAMP results. None of the LAMP-positive/CCNA-negative cases had antibiotic or
antineoplastic exposure. All three experienced symptom resolution without CDI therapy. In
addition, one case had a positive Rotavirus antigen EIA test, and a second case had Blastocystis
hominis in his stool. All three CCNA-positive/LAMP-negative cases had risk factors for CDI
(exposure to antineoplastic agents in one, beta-lactam antibiotics in the other two, within 8 weeks
of the positive CCNA result.) One case responded clinically to metronidazole therapy and
therefore appeared to be a true CDI case. The other two tested positive for Norovirus by PCR,
providing an alternative diagnosis, making CDI less likely but still possible. All three patients
had negative stool cultures.

Discussion

In our pediatric cohort, CCNA and LAMP alone performed with the highest test
sensitivity as standalone tests. The GDH-based algorithms performed with lower sensitivity
(81%) compared to adult studies in the literature (98-100%), suggesting that this approach may
fail to diagnose 19% of pediatric patients with CDI (10, 12, 15). The reason for the lower
sensitivity is unclear. It has been suggested that GDH antigen-based assays may demonstrate
superior performance in populations with high incidence of CDI mediated by hypervirulent C.
difficile strains (16). Indeed, ribotyping of a small series of C. difficile isolates from our
institution in 2007 indicated that the prevalence of the NAP1/027/III strain among P-EIA-
positive stool specimens was only 14% (17). We note, however, that the sensitivity of the GDH-
based algorithms was still statistically superior to P-EIA.
In this study, we observed a PPV of 100% for GDH-positive/CDT-positive specimens and an NPV of 95% for GDH-negative/CDT-negative specimens, strongly supporting the practice of reporting these specimens as “positive” or “negative” for *C. difficile* toxin immediately without further testing. 80% of all specimens tested in our cohort were immediately reportable following GDH/CDT testing (8% were GDH-positive/CDT-positive and 72% were GDH-negative/CDT-negative). The implications of the rapid GDH/CDT reporting are significant in our population. While syndromic infection control precaution assignment (13) is the current standard in our institution when the etiology of an inpatient with diarrhea has yet to be determined (i.e. a child with diarrhea will be isolated with contact precautions while symptomatic regardless of *C. difficile* toxin status), our data indicate that about half of all CDI cases would be rapidly reported following GDH/CDT testing. With a 19% CDI prevalence, we anticipate that timelier initiation of CDI therapy and necessary environmental cleaning and disinfection procedures in even half of all CDI cases would be beneficial for the control of CDI in our patient population. Moreover, while rapid reporting of negative results may not affect resources allocated to the application of contact precautions on symptomatic patients, it may facilitate cohorting of inpatients and encourage clinicians to consider other etiologic causes of antibiotic-associated diarrhea.

In our cohort, the PPV of 52% for GDH alone confirmed the need to pursue additional testing of GDH-positive/CDT-negative specimens to resolve these specimens as “positive” or “negative” for *C. difficile* toxin. PPV of 100% and NPV of 96% were observed for the entire algorithm when CCNA and LAMP were used in this capacity, indicating that both algorithmic approaches performed well in our population. The meaning of positive molecular CDI assays has been questioned in some quarters on the grounds that they do not detect *C. difficile* toxin or
even the presence of organisms with the capacity to produce toxin in vitro but merely
demonstrate the presence of the genes that code for toxins A and B (tcdA and tcdB) (18). In our
study, the virtually identical performance of CCNA and LAMP-confirmed algorithms provided
reassurance that the user-friendly and relatively rapid LAMP procedure was as appropriate as
CCNA in a confirmatory role. LAMP performed with sensitivity and specificity comparable to
other studies evaluating this assay (7, 9).

With regard to discordance, LAMP did not detect three specimens that were CCNA-
positive. Chart review suggested that at least one of these cases was consistent with CDI,
suggesting that the LAMP result may have been falsely negative. From the assay standpoint, the
illumigene C. difficile assay uses an internal control to detect LAMP reaction failures related to
inhibition reagent problems. Failure of the internal control reaction is indicated by an “Invalid”
test result. All three CCNA-positive, LAMP-negative results yielded valid “Negative” results,
indicating that the LAMP reactions were successful in each case. With respect to target design,
while the illumigene C. difficile assay’s tcdA (as opposed to tcdB) target has raised questions
about its ability to detect toxin A− B+ strains resulting from tcdA mutations, the target sequence
appears to be conserved (5). Limited data demonstrating this assay’s ability to detect these
strains have been presented, supporting the manufacturers’ assertion that this assay has the
capacity to detect toxin A− strains (5). Mutation at the actual LAMP target site, however, needs
to be entertained as a possible explanation for these cases (5). Finally, false negative LAMP
results in specimens that were positive by toxigenic culture have been documented in the
literature (7). While there are reports in the literature of inferior sensitivity of CCNA compared
to LAMP (7, 9), the sensitivity of CCNA is highly dependent on a number of factors including
the quality of the cell line used and the time interval between specimen collection and
inoculation of the cell culture plates (6). We feel that when carefully performed, CCNA may
detect CDI with a sensitivity that compares with molecular techniques. While to our knowledge,
CCNA-positive/LAMP-negative specimens have not been reported in the literature, this
phenomenon has been described with other commercial molecular assays (6) and may be
explained by the presence of low bacterial concentrations of hypervirulent strains of toxigenic C. 
difficile producing disproportionately high levels of cytotoxin. Unfortunately, we did not have
the means to pursue molecular typing of C. difficile isolates to investigate this hypothesis further.

There were three LAMP-positive/CCNA-negative specimens. Chart review suggested
that these cases did not have CDI. None of the cases had clinical risk factors for CDI and all
three cases experienced symptom resolution without CDI therapy. In addition, two cases
appeared to have alternative diagnoses. Possible false positive LAMP results (using toxigenic
culture as the gold standard) have been reported in the literature. Norén et al. described four such
cases, with only one confirmed with an in-house PCR assay (9). End product detection in loop-
mediated isothermal amplification technology relies on pyrophosphate ion released from dNTP
during DNA polymerization reacting with magnesium ion to produce a precipitate. This is
followed by detection of the resulting increase in turbidity of the reaction mixture (8). We
speculate that false positive LAMP results may be related to non-specific DNA amplification
generating magnesium pyrophosphate or substances in stool specimens causing increases in
turbidity of the reaction mixture.

This study’s potential issue of controversy is the omission of toxigenic culture in our gold
standard definition. The optimal gold standard method remains an issue of contention (18).
While the SHEA-IDSA guidelines support the use of toxigenic culture for comparative studies
(2), discussion in the literature continues to debate the merits of toxin detection versus the
detection of *C. difficile* isolates with in-vitro capacity to produce toxin (18). In this study, we opted to use CCNA as opposed to toxigenic culture on the basis that detection of toxin (the “sine qua non for CDI”) is preferable (18). Also, while there are data suggesting that correlation between toxigenic culture and molecular testing is superior to CCNA with the latter (15), evidence also suggests that specimens that are negative by GDH/CDT and CCNA but positive by PCR and toxigenic culture may represent colonization with toxigenic *C. difficile* as opposed to actual CDI (6).

In summary, an algorithmic approach to CDI detection with the *C. DIFF QUIK CHEK COMPLETE* product for initial CDI testing of pediatric stool specimens allowed swift reporting of 80% of *C. difficile* toxin results while increasing diagnostic yield over P-EIA in a tertiary pediatric population. The *illumi*gene *C. difficile* assay provided an operator-friendly and accurate method of resolving GDH-positive/CDT-negative results. The sensitivity of the *C. DIFF QUIK CHEK COMPLETE*, however, may not be sufficient to allow its use as a standalone “rule-out” test in a pediatric population.

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References


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Clostridium difficile infection in adults: 2010 update by the Society for Health
care Epidemiology of America (SHEA) and the Infectious Diseases Society of

commercially available Clostridium difficile toxin detection assays, a real-time PCR
assay for C. difficile tcdB, and a glutamate dehydrogenase detection assay to cytotoxin


appropriate for detection of A and/or B Clostridium difficile toxin-producing strains?

GeneOhm Cdiff real-time PCR assay with a two-step algorithm and a toxin A/B enzyme-
linked immunosorbent assay for diagnosis of toxigenic Clostridium difficile infection. J.


Preventing Transmission of Infectious Agents in Healthcare Settings.


Table 1. Performance of four *C. difficile* toxin assays in a tertiary pediatric population

<table>
<thead>
<tr>
<th>Gold standard</th>
<th>TP</th>
<th>TN</th>
<th>FP</th>
<th>FN</th>
<th>SN</th>
<th>SP</th>
<th>PPV</th>
<th>NPV</th>
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<tr>
<td>GDH^1</td>
<td>22</td>
<td>94</td>
<td>20</td>
<td>5</td>
<td>81%</td>
<td>82%</td>
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<td>95%</td>
</tr>
<tr>
<td>CCNA^2</td>
<td>21</td>
<td>94</td>
<td>21</td>
<td>5</td>
<td>81%</td>
<td>82%</td>
<td>50%</td>
<td>95%</td>
</tr>
<tr>
<td>CDT^2</td>
<td>13</td>
<td>114</td>
<td>0</td>
<td>14</td>
<td>48%</td>
<td>100%</td>
<td>100%</td>
<td>89%</td>
</tr>
<tr>
<td>EIA^3</td>
<td>15</td>
<td>114</td>
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<td>12</td>
<td>56%</td>
<td>100%</td>
<td>100%</td>
<td>90%</td>
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<td>CCNA^4</td>
<td>15</td>
<td>115</td>
<td>0</td>
<td>11</td>
<td>58%</td>
<td>100%</td>
<td>100%</td>
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</tr>
<tr>
<td>CCNA</td>
<td>26</td>
<td>114</td>
<td>0</td>
<td>1</td>
<td>96%</td>
<td>100%</td>
<td>100%</td>
<td>99%</td>
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<td>LAMP^5</td>
<td>24</td>
<td>111</td>
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<td>97%</td>
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<td>0</td>
<td>5</td>
<td>81%</td>
<td>100%</td>
<td>100%</td>
<td>96%</td>
</tr>
<tr>
<td>CCNA</td>
<td>21</td>
<td>115</td>
<td>0</td>
<td>5</td>
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<td>100%</td>
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<tr>
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<td>100%</td>
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<td>5</td>
<td>81%</td>
<td>100%</td>
<td>100%</td>
<td>96%</td>
</tr>
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</table>
1GDH, *C. DIFF QUIK CHEK COMPLETE* glutamate dehydrogenase antigen enzyme immunoassay

2CDT, *C. DIFF QUIK CHEK COMPLETE* *C. difficile* toxin A and toxin B immunoassay

3EIA, Premier *C. difficile* Toxin A & B enzyme immunoassay

4CCNA, Cell cytotoxicity neutralization assay

5LAMP, Loop-mediated isothermal amplification

6Algorithm #1: Initial testing with *C. DIFF QUIK CHEK COMPLETE*, followed by LAMP for GDH-positive/CDT-negative specimens

7Algorithm #2: Initial testing with *C. DIFF QUIK CHEK COMPLETE*, followed by CCNA for GDH-positive/CDT-negative specimens

8Composite gold standard: a specimen was considered “True Positive” if positive by CCNA. If the specimen was CCNA-negative, the specimen would also be “True Positive” if LAMP-positive and positive by another test (GDH, CDT or EIA).

9CCNA gold standard: a specimen was considered “True Positive” if positive by CCNA.

10TP, True positive

11TN, True negative

12FP, False positive

13FN, False negative

14SN, Sensitivity
15SP, Specificity
16PPV, Positive predictive value
17NPV, Negative predictive value