Detection of Toxigenic Clostridium difficile: Comparison of the Cell Culture Neutralization, Xpert® C. difficile, Xpert® C. difficile/Epi and the Illumigene™ C. difficile Assays

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Abstract

*Clostridium difficile* (CD) is the most important cause of nosocomial diarrhea. Several laboratory techniques are available to detect CD toxins or the genes that encode them in fecal samples. We evaluated the Xpert® CD and Xpert® CD/Epi (Cepheid, CA) that detect the toxin B gene (*tcdB*), or *tcdB*, *cdt*, and a deletion in *tcdC* associated with the 027/NAP1/BI strain, respectively, by real time PCR, and the Illumigene™ CD (Meridian Bioscience, Inc.) that detects the toxin A gene (*tcdA*) by loop-mediated isothermal amplification in stool specimens. Toxigenic culture was used as the reference method for discrepant stool specimens. Two hundred prospective and 50 retrospective diarrheal stool specimens were tested simultaneously by the cell cytotoxin neutralization assay (CCNA), Xpert CD, Xpert CD/Epi, and Illumigene CD assays. Of the 200 prospective stools tested, 10.5% (n=23) were positive by CCNA, 17.5 % (n=35) by Illumigene CD, and 21.5% (n=43) by Xpert CD and Xpert CD/Epi. Of the 50 retrospective stools, previously positive by CCNA, 94% (n=47) were positive by Illumigene CD and 100 % (n=50) by Xpert CD and Xpert CD/Epi. Of the 11 discrepant results (negative by Illumigene CD but positive by Xpert CD and Xpert CD/Epi), all were positive by the toxigenic culture. Twenty-one percent of isolates were presumptively identified by the Xpert CD/Epi as the 027/NAP1/BI strain. The Xpert CD and Xpert CD/Epi assays were the most sensitive, rapid, and easy to use assays for the detection of toxigenic CD in stool specimens.
Clostridium difficile (CD) is responsible for the majority of cases of infectious antibiotic-associated diarrhea and pseudomembranous colitis and is rapidly increasing in prevalence (3, 4). CD infection (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 (15). 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 (24). Many hospitals are now required to report health care-associated transmission of pathogens, including CD, to public health departments. Thus, it is imperative that the diagnosis of CDI be rapid and accurate.

The pathogenic effects of CD are mucosal damage to the colon that is caused by toxin A and/or toxin B. The diagnostic methods that target one or both of these toxins include enzyme immunoassay (EIA) and cell culture neutralization assay (CCNA), performed on stool samples. Although the various EIA methods have proven to be less than optimal diagnostic tests, they are the assays that are most commonly used (5, 16). EIA methods offer a rapid turnaround time (TAT), compared with CCNA or culture for toxigenic CD organisms—tests for which the time to final result can be 48–72 h. However, EIA is associated with widely varying sensitivity (50%–99%) and specificity (70%–100%) with performance largely dependent on which reference method is used for comparison making its reliability questionable for an accurate diagnosis of CDI (9). Several nucleic acid amplification tests (NAATs) are FDA-cleared for CD testing, and, compared to other non-culture-based methods, NAATs are the most sensitive methods available (9). However, the platforms and ease of use vary considerably. These assays detect
conserved regions of toxin A (tcdA) or tcdB genes located on the PaLoc of CD (2, 6, 10-12, 17, 24).

Several hypervirulent strains responsible for the global epidemics have been described, the most widespread of which is the isolate designated by pulsed-field gel electrophoresis (PFGE) as the North American profile 1 (NAP1), or as toxinotype III, PCR-ribotype 027, or restriction endonuclease analysis type BI (14, 23). The 027/NAP1/BI strain produces increased levels of toxin A and toxin B (25), a third toxin called the binary toxin, and also carries an 18 base pair (bp) deletion and a 1-bp deletion (at nucleotide [nt] 117) in the tcdC gene, a putative negative regulator of tcdA and tcdB gene expression (13, 14). For epidemiological studies, positive CD isolates are further analyzed by PFGE, PCR-ribotyping and/or direct sequencing of the tcdC gene to detect the 18 bp or nucleotide 117 deletions (22).

The goal of the current study was to compare the performance of the Xpert CD and Xpert CD/Epi (Cepheid, CA) that detect the tcdB by real time PCR and the Illumigene™ CD (Meridian Bioscience, Inc.) that detects the tcdA by loop-mediated isothermal amplification assays (LAMP) to the CCNA for the rapid, sensitive and specific detection of toxigenic CD in stool specimens.

Material and Methods

Specimens. A total of 250 stool specimens (200 prospective and 50 retrospective) collected from adult patients (age >18 years) were tested in this study. Specimens
tested were unformed stools (defined as a room temperature specimen that took the form of the collection container) submitted to The Ohio State University Medical Center (OSUMC) Clinical Microbiology Laboratory for routine CDI diagnosis between March, 2010 and December, 2010. Duplicate specimens from the same patients were excluded. Prospective specimens included sequential stools that were tested daily or stored at 4°C and tested within 24 hours (h). The 50 stool specimens collected retrospectively were based on a positive CCNA. The latter stools were frozen at -70 °C until use.

**Xpert CD PCR assay.** The Xpert CD assay is a real-time PCR that detects tcdB. The assay 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 seconds (s), and all the liquid from the sample reagent was transferred to the "S" chamber of the cartridge using a large transfer pipette. Next, reagent 1 was added to chamber 1 of the test cartridge. Finally, reagent 2 was added to chamber 2 of the test cartridge, and the lid was closed. The cartridge barcode was scanned and placed in the GeneXpert instrument. Results were reported as positive, negative or invalid.

**Xpert CD/Epi PCR assay.** The Xpert CD/Epi assay is a multiplex real-time PCR that detects tcdB, the binary toxin gene (cdt), and the tcdC gene deletion at nt 117. The assay was performed according to the manufacturer's instructions. Briefly, a stool sample was collected on a swab (Cepheid collection device) from the container received in the laboratory and transferred into the sample reagent vial. The vial was vortexed for 10 s and the solution pipetted into the “S” chamber of the cartridge by using a Pasteur pipette. The cartridge was then placed on the GeneXpert instrument, and the test was
performed using the GeneXpert CD assay program. Potential results included the following: toxigenic CD positive/presumptive 027/NAP1/BI negative, toxigenic CD positive/presumptive 027-NAP1-BI positive, toxigenic CD negative/presumptive 027/NAP1/BI negative, invalid, error, or no results.

**Illumigene CD LAMP assay.** The Illumigene CD assay is based on loop-mediated amplification technology. The assay targets a conserved 204-bp sequence within the\textit{tcdA} region of the CD pathogenicity locus (PaLoc; 17). The Illumigene CD assay was performed according to the manufacturer's instructions. Briefly, the stool specimen collected on an Illumigene sample brush was added to a sample preparation apparatus containing sample diluents. The sample was vortexed for 10 s and 5-10 drops of the sample were squeezed into a clean Illumigene extraction tube. The sample tube was heated in a heat block at 95°C for 10 minutes after which the tube was vortexed. The extracted sample (50 µl) was transferred to an Illumigene reaction buffer tube and vortexed for 10 s. Using a new pipette tip, 50ul was transferred from the reaction buffer tube to the test chamber and control chamber of the Illumigene assay device that contains the appropriate beads (white and yellow) respectively. The Illumigene assay device was then inserted into the illumipro-10 to initiate the amplification reaction and detection. Results were reported as positive, negative or invalid. Testing of specimens with an invalid result was repeated once.

**CCNA.** Stool specimens were diluted (1:3) in Hanks Balanced Salt Solution and centrifuged for 20 minutes at 3,100 rpm. The resulting supernatants were filtered (0.22-µm pore size), 50 µl of filtrate was added to skin fibroblasts cells (96-well microtiter
plate; Quidel, Athens, OH) and incubated for 48h at 37°C, 5% CO₂. To control for nonspecific toxicity, a second well was inoculated with both the supernatant and 50 µl of CD goat antitoxin (TECHLAB, Blacksburg, VA). The cells were incubated at 37°C and checked for cytopathic effect (CPE) at 6h, 22h, 30h and 48h. A positive result was defined as the presence of CPE in at least 50% of the cell monolayer and no CPE in the tube inoculated with the antitoxin.

**Toxigenic culture:** Anaerobic culture was performed on discrepant stool specimens by plating specimens onto prereduced cycloserine-cefoxitin-fructose agar media (CCFA-VA formulation; Remel, Lenexa, KS). Plates were incubated anaerobically using the anaerobe chamber (Bactron IV-Sheldon Manufacturing) at 35°C for up to 5 days before a final interpretation of a negative result was determined. Identification of CD was achieved by characteristic yellow spready colonies, yellow–green fluorescence under ultra violet light, negative indole reaction, and a positive L-proline aminopeptidase (Remel) reaction (8). A score of 99.99% was obtained on the Rapid Anaerobe ID Panel (Siemens Healthcare Diagnostics, Deerfield, IL).

The CD isolates were grown for 24h in anaerobic Brucella broth (Remel), and supernatant passed through a 0.22-µm filter (Spin-X centrifuge tube filter; Millipore, Billerica, MA) was used to determine toxigenicity as described above for CCNA.

**Confirmation of 027/NAP1/BI:** The 027/NAP1/BI positive result by the Xpert CD/Epi assay was confirmed by toxinotyping PFGE (25), and/or by sequencing (22) which identified the isolate as the epidemic strain 027/NAP1/BI (toxinotype III; binary toxin positive; 18-bp tcdC deletion).
Discrepant resolution: Anaerobic toxigenic culture was used as the reference standard method for discrepant analysis. A specimen was considered discrepant if even one assay (NAAT or CCNA) was not in agreement with the other assay results. In the latter case, toxigenic culture was utilized as the reference method.

Statistics
Result concordance of the four assays was used as the reference standard for sensitivity and specificity calculations. Toxigenic culture was only used to resolve discrepancies and was not performed for all specimens.

Results
The performance of the Xpert CD and Xpert CD/Epi (Xpert CD assays) and Illumigene CD assay were assessed in 250 stool specimens. The results of each assay were compared to the results of the CCNA. The discrepant specimens were tested by the toxigenic culture.

Of the 200 prospective stool specimens, 157 were negative by the CCNA and the NAAT assays (Table 1), 20 specimens were positive by all the 4 assays; and 23 specimens gave discrepant results for the CCNA and NAAT tests. Four samples were positive by Xpert CD but negative by both CCNA and Illumigene CD assays; all 4 were positive by toxigenic culture. Three specimens were positive by the Xpert CD assays and CCNA but were negative by the Illumigene CD assay; all three were also positive by toxigenic culture. One sample was invalid with the Illumigene CD assay, but positive by Xpert
Overall, 23 (10.5%) specimens were positive by the CCNA as compared to 35 (17.5%) and 43 (21.5%) by the Illumigene CD and Xpert CD assays respectively (Table 1). The NAAT tests and CCNA showed a specificity of 100%. The Xpert CD assays showed the highest sensitivity (100%) followed by Illumigene CD (83%) and CCNA (54%). Of the 8 discrepant results between the Illumigene CD and the Xpert CD assays, all were confirmed to be positive by the toxigenic culture and two of the discrepant specimens were also positive for 027/NAP1/BI (Table 2).

For retrospective stool specimens, all 50 (100%) CCNA positive were also positive by the Xpert CD assays. In contrast, 3/50 (6%) specimens were negative by the Illumigene CD assay, 2 of which were 027/NAP1/BI strains (Table 2). The NAAT tests and CCNA showed a specificity of 100%. The Xpert CD assays and the CCNA showed the highest sensitivity (100%) compared to Illumigene CD (94%). Of the 3 discrepant specimens, all were positive by the toxigenic culture and two were also positive for 027/NAP1/BI (Table 2). Of the 43 prospective tcdB positive specimens by the Xpert CD assays, the Xpert CD/Epi assay reported 2 (5%) as positive for tcdB and cdt; 32 (74%) were positive for tcdB alone, and 9 (21%) were positive for tcdB, tcdC deletion, and cdt. The latter were reported as presumptive 027/NAP1/BI (Table 3). For the retrospective CD positive stool specimens, the Xpert CD/Epi assay reported 4 CD as positive for tcdB and cdt; 19 were positive for tcdB alone, and 27 were positive for tcdB, tcdC deletion, and cdt. The latter were reported as presumptive 027/NAP1/BI (Table 3). The results for the 027/NAP1/BI strains were confirmed by PFGE and/or tcdC gene sequencing (data not shown).
The laboratory diagnosis of CDI continues to be challenging. The latest guidelines from the Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America reemphasized the need to consider two-step algorithms that use glutamate dehydrogenase (GDH) assays to screen for CD in stool specimens, followed by either CCNA testing, toxigenic culture, or NAAT to identify toxin-producing CD isolates (4). While early studies comparing the GDH assay to CCNA demonstrated high sensitivity and negative predictive values, more recent comparisons to toxigenic culture and PCR have shown the sensitivity to be in the 71-100% range (9, 19, 20). In addition, the two-step approach can cause a delay of 48 to 92 hours that would require contact isolation for patients with suspected CDI until the testing is complete.

At this time, 4 FDA cleared nucleic acid amplification assays are available to clinical laboratories and several of these have been well evaluated in the literature (2, 6, 7, 26). The Illumigene CD assay uses loop-mediated isothermal amplification technology to detect tcdA in the pathogenicity locus of toxigenic CD. The test includes a manual extraction step but does not require costly capital equipment, and results are available in approximately 1h. The Xpert CD, BD GeneOhm CD (BD Diagnostics, La Jolla, CA), and proGastro CD (Gen-Probe Prodesse, Inc., Waukesha, WI) assays are based on real-time PCR and target tcdB. The reported sensitivities of the assays vary from 91.7% to 95.2% respectively, with specificities of 94% and 95.5%, respectively (7). Because the assays detect the tcdA or the tcdB genes and not the actual toxins, the enhanced sensitivity of these amplification methods, testing of CD should be limited to patients with clinical symptoms of CDI (6).
In the present study, we compared the sensitivity and specificity of the CCNA with those of the Xpert CD assays and the Illumigene CD assay. While both the Xpert CD and the Illumigene CD assays showed greater sensitivity and quicker TAT (45 minutes and 1h respectively) as compared to the CCNA (median TAT for the positive specimens was 24 hours (range 6 to 72h), the Xpert CD assays were more sensitive than the Illumigene CD assay. In the prospective arm of the study, overall 10.5% specimens were positive by the CCNA as compared to 17.5% and 21.5% by the Illumigene CD and Xpert CD assays respectively (Table 1). The 027/NAP1/BI prevalence was 21%. The toxigenic culture of discrepant specimens showed the Xpert CD assays to have detected 24 (100%), and the Illumigene CD assay to have detected 17 (71%) of 24 true positives. Overall, the Xpert CD assays detected 8 additional CD positive specimens three of which were also CCNA positive (Table 1).

In addition to the \textit{tcdB}, the Xpert CD/Epi presumptively identifies the 027/NAP1/BI strain by detecting the binary toxin gene, and the \textit{tcdC} nt 117 gene deletion. All 027/NAP1/BI strains identified were positive for all three markers (Table 3). The positive results for 027NAP1/BI by the Xpert CD/Epi were confirmed with PFGE (25) and/or sequencing of the \textit{tcdC} gene (22). All nine strains identified were positive for all three markers (Table 3). Agreement between the above methods was 100% (data not shown). One 027/NAP1/BI positive specimen was resulted as invalid by the Illumigene CD assay. Upon close examination, the invalid specimen contained visible blood which could have contributed to inhibition.
In the retrospective arm of the study, 100% of the previously CCNA positive specimens were positive by the Xpert CD assays, while only 47 out of 50 specimens were positive by the Illumigene CD assay (Table 2). Of the 3 specimens not detected by the Illumigene CD assay, 2 specimens were 027/NAP1/BI positive (Table 2). While the reason for low sensitivity of the Illumigene CD assay is not clear, we speculate that organism load, mutations or polymorphisms in primer- or probe-binding regions may affect detection of CD tcdA variants, resulting in false-negative results.

The lack of detection of the tcdA in 027/NAP1/BI positive strains by the Illumigene CD assay is of concern. The 027/NAP1/BI strain is responsible for widespread outbreaks of CD in North America (13, 14, 27). The hypervirulent strains have been reported to exhibit increased sporulation capacity along with high levels of toxin production (1, 25). The significance of 027/NAP1/BI result as an epidemiological marker is known and outbreaks caused by a toxin variant epidemic strain have renewed interest in detecting this strain. With regards to 027/NAP1/BI as a marker for disease severity, the increased severity and mortality of 027/NAP1/BI strains are of particular concern for infection prevention in a healthcare setting (13). These strains are associated with both community acquired and healthcare associated CDI. However, a preliminary review of patients with and without the 027/NAP1/BI strain did not show any significant differences in disease severity (21). Thus, this issue requires further investigation. Of note, one recent study has indicated that the presence of binary toxin may be an independent risk factor for increased disease severity and mortality, independent of strain type (13).
The Xpert CD assays had the highest sensitivity of the assays investigated in our study; the assay detected all potential positive results as confirmed by the toxigenic culture. Perhaps most importantly for the accurate diagnosis of this infectious disease is the fact that the rapid, real-time PCR assay had sensitivity similar to that of culture for detecting toxigenic CD, while retaining the specificity of the direct cytotoxicity test. In a study by Novak Weekly et al, the Xpert CD testing yielded the highest sensitivity and negative predictive value in the least amount of time, of the individual- and multiple-test algorithms evaluated (18).

The reagent cost of each assay and the amount of technical time required was $46 and 4 minutes, respectively, for Xpert CD; $26 and 5 minutes, respectively, for the Illumigene CD assay and $12 and 5 minutes, respectively, for CCNA; and $27 and 30 minutes, respectively, for anaerobic culture. Despite the higher cost, the greatest impact of adopting the Xpert CD assays will be in the value in effectively reducing the time patients are kept in isolation. Results can be obtained by real-time PCR closed walk-away systems more rapidly than by more traditional PCR assays (26). Some of the limitations in our study include the use of the gold standard toxigenic culture only in cases where results were discrepant between Illumigene CD, Xpert CD assays and CCNA. This approach, could potentially affect the overall sensitivity and specificity of the two assays tested.

In conclusion, the Xpert CD assays were more sensitive for detection of toxigenic CD and for the laboratory confirmation of CDI as compared to the Illumigene CD assay.
Competing interests

None.

Acknowledgements

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Table 1: Comparison of Xpert CD, Xpert CD/Epi, Illumigene CD, CCNA and Toxigenic Culture in Prospective Stool Specimens

<table>
<thead>
<tr>
<th># Specimens</th>
<th>Xpert CD &amp; Xpert CD/Epi</th>
<th>Illumigene CD</th>
<th>CCNA</th>
<th>Toxigenic Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>157</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Not performed</td>
</tr>
<tr>
<td>20</td>
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<td>Positive</td>
<td>Positive</td>
<td>Not performed</td>
</tr>
<tr>
<td>15</td>
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<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>4</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>3</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>1</td>
<td>Positive</td>
<td>Invalid</td>
<td>Negative</td>
<td>Positive</td>
</tr>
</tbody>
</table>

1 Results for both assays were identical
Table 2: Discrepant analysis of stool specimens between Xpert CD, Xpert CD/Epi and Illumigene CD assays with toxigenic culture

<table>
<thead>
<tr>
<th></th>
<th>Xpert CD &amp; Xpert CD/Epi</th>
<th>Illumigene CD</th>
<th>CCNA</th>
<th>Toxigenic culture</th>
<th>Presumptive 027/NAP1/BI</th>
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</thead>
<tbody>
<tr>
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<td>Negative</td>
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<td>Negative</td>
</tr>
<tr>
<td>P2</td>
<td>Positive</td>
<td>INVALID</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive*</td>
</tr>
<tr>
<td>P3</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive*</td>
</tr>
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<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
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<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Negative</td>
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<td>Positive</td>
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</tr>
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<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
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<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
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<td>Positive</td>
<td>Positive*</td>
</tr>
<tr>
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<td>Positive</td>
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<td>Positive</td>
<td>Positive</td>
<td>Positive*</td>
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</tbody>
</table>

P: Prospective stool specimens; R: Retrospective stool specimens

* 027/NAP1/BI results confirmed by PFGE and tcdC sequencing
Table 3: Presumptive identification of 027/NAP1/BI strain by the Xpert CD/Epi Assay in Toxin B positive stool specimens

<table>
<thead>
<tr>
<th>Prospective Specimens</th>
<th>Retrospective Specimens</th>
<th>tcdB</th>
<th>tcdC deletion</th>
<th>cdt</th>
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<tbody>
<tr>
<td>2</td>
<td>4</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>32</td>
<td>19</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9 (027/NAP1/BI)</td>
<td>27 (027/NAP1/BI)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>