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

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Clostridium difficile **is the most important cause of nosocomial diarrhea. Several laboratory techniques are available to detect *C. difficile* toxins or the genes that encode them in fecal samples. We evaluated the Xpert *C. difficile* and Xpert *C. difficile*/Epi (Cepheid, CA) that detect the toxin B gene (*tcdB*) and *tcdB*, *cdt*, and a deletion in *tcdC* associated with the 027/NAP1/BI strain, respectively, by real-time PCR, and the Illumigene *C. difficile* (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 fifty retrospective diarrheal stool specimens were tested simultaneously by the cell cytotoxin neutralization assay (CCNA) and the Xpert *C. difficile*, Xpert *C. difficile*/Epi, and Illumigene *C. difficile* assays. Of the 200 prospective stools tested, 10.5% ($n = 23$) were determined to be positive by CCNA, 17.5% ($n = 35$) were determined to be positive by Illumigene *C. difficile*, and 21.5% ($n = 43$) were determined to be positive by Xpert *C. difficile* and Xpert *C. difficile*/Epi. Of the 50 retrospective stools, previously determined to be positive by CCNA, 94% ($n = 47$) were determined to be positive by Illumigene *C. difficile* and 100% ($n = 50$) were determined to be positive by Xpert *C. difficile* and Xpert *C. difficile*/Epi. Of the 11 discrepant results (i.e., negative by Illumigene *C. difficile* but positive by Xpert *C. difficile* and Xpert *C. difficile*/Epi), all were determined to be positive by the toxigenic culture. A total of 21% of the isolates were presumptively identified by the Xpert *C. difficile*/Epi as the 027/NAP1/BI strain. The Xpert *C. difficile* and Xpert *C. difficile*/Epi assays were the most sensitive, rapid, and easy-to use assays for the detection of toxigenic *C. difficile* in stool specimens.**

Clostridium difficile is responsible for the majority of cases of infectious antibiotic-associated diarrhea and pseudomembranous colitis and is rapidly increasing in prevalence (3, 4). *C. difficile* 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 *C. difficile*, to public health departments. Thus, it is imperative that the diagnosis of CDI be rapid and accurate.

The pathogenic effects of *C. difficile* 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, these are the assays that are most commonly used (5, 16). EIA methods offer a rapid turnaround time (TAT) compared to CCNA or culture for toxigenic *C. difficile* organisms, tests for which the time to final result can be 48 to 72 h. However, EIA is associated with widely varying sensitivity (50 to 99%) and specificity (70 to 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 U.S. Food and Drug Administration (FDA)-cleared for *C. difficile* 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 B (*tcdB*) genes located on the pathogenicity locus (PaLoc) of *C. difficile* (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) and a third toxin, called the binary toxin, and it also carries an 18-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 *C. difficile* isolates are further analyzed by PFGE, PCR-ribotyping, and/or direct sequencing of the *tcdC* gene to detect the 18-bp or nt 117 deletions (22).

The goal of the present study was to compare the performance of the Xpert *C. difficile* and Xpert *C. difficile*/Epi assays (Cepheid, CA) that detect the *tcdB* by real-time PCR and the Illumigene *C. difficile* assay (Meridian Bioscience, Inc.) that detects the *tcdA* by loop-mediated isothermal amplification (LAMP) assays to the

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CCNA for the rapid, sensitive, and specific detection of toxigenic *C. difficile* in stool specimens.

MATERIALS AND METHODS

Specimens. A total of 250 stool specimens (200 prospective and 50 retrospective) collected from adult patients (>18 years old) were tested in the present study. The 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 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 h. The 50 stool specimens collected retrospectively were based on a positive CCNA. The latter stools were frozen at −70°C until use.

Xpert *C. difficile* PCR assay. The Xpert *C. difficile* 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 s, and all of 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 an Xpert instrument. The results were reported as positive, negative, or invalid.

Xpert *C. difficile*/Epi PCR assay. The Xpert *C. difficile*/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 Xpert instrument, and the test was performed using the GeneXpert *C. difficile* assay program. Potential results included the following: toxigenic *C. difficile* positive/presumptive 027/NAP1/BI negative, toxigenic *C. difficile* positive/presumptive 027-NAP1-BI positive, toxigenic *C. difficile* negative/presumptive 027/NAP1/BI negative, invalid, error, or no results.

Illumigene *C. difficile* LAMP assay. The Illumigene *C. difficile* assay is based on LAMP technology. The assay targets a conserved 204-bp sequence within the *tcdA* region of the PaLoc (17). The Illumigene *C. difficile* 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 to 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 min, 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, 50 μl 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 an Illumipro-10 (Meridian Bioscience, Inc.) to initiate the amplification reaction and detection. The 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 min at 3,100 rpm. The resulting supernatants were filtered (0.22-μm pore size), and 50 μl of filtrate was added to skin fibroblasts cells (96-well microtiter plate; Quidel, Athens, OH), followed by incubation for 48 h at 37°C and 5% CO_2. To control for nonspecific toxicity, a second well was inoculated with both the supernatant and 50 μl of *C. difficile* goat antitoxin (Techlab, Blacksburg, VA). The cells were incubated at 37°C and checked for cytopathic effect (CPE) at 6, 22, 30, and 48 h. 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). The plates were incubated anaerobically using an 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 *C. difficile* was achieved by characteristic yellow flat colonies, yellow-green fluorescence under UV light, a 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 *C. difficile* isolates were grown for 24 h in anaerobic brucella broth (Remel), and supernatant passed through a 0.22-μm-pore-size filter (Spin-X centrifuge tube filter; Millipore, Billerica, MA) was used to determine the toxigenicity as described above for the CCNA.

Confirmation of 027/NAP1/BI. The 027/NAP1/BI positive result by the Xpert *C. difficile*/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) result 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 *C. difficile* and Xpert *C. difficile*/Epi (Xpert *C. difficile* assays) and Illumigene *C. difficile* assays was 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 determined to be negative by the CCNA and the NAAT assays (Table 1), 20 specimens were determined to be positive by all four assays, and 23 specimens gave discrepant results for the CCNA and NAAT tests. Four samples were positive by the Xpert *C. difficile* assay but negative by both CCNA and Illumigene *C. difficile* assays; all four were positive by toxigenic culture. Three specimens were positive by the Xpert *C. difficile* assays and CCNA but negative by the Illumigene *C. difficile* assay; all three were also positive by toxigenic culture. One sample was invalid using the Illumigene *C. difficile* assay but positive by Xpert *C. difficile* assay, CCNA, and toxigenic culture. Overall, 23 (10.5%) specimens were determined to be positive by the CCNA compared to 35 (17.5%) and 43 (21.5%) by the Illumigene *C. difficile* and Xpert *C. difficile* assays, respectively (Table 1). The NAAT and CCNA showed a specificity of 100%. The Xpert *C. difficile* assays showed the highest sensitivity (100%), followed by the Illumigene *C. difficile* assay (83%) and CCNA (54%). Of the eight discrepant results between the Illumigene *C. difficile* and Xpert *C. difficile* assays, all were confirmed to be positive by 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 specimens were also determined to be positive by the Xpert *C. difficile* assays. In contrast, 3/50 (6%) specimens were negative by

TABLE 1 Comparison of Xpert *C. difficile*, Xpert *C. difficile*/Epi, and Illumigene *C. difficile* assays, CCNA, and toxigenic culture in prospective stool specimens

Specimen group[a]	No. of specimens	Test result			
		Xpert *C. difficile* and Xpert *C. difficile*/Epi[b]	Illumigene *C. difficile*	CCNA	Toxigenic culture
1	157	Negative	Negative	Negative	Not performed
2	20	Positive	Positive	Positive	Not performed
3	15	Positive	Positive	Negative	Positive
4	4	Positive	Negative	Negative	Positive
5	3	Positive	Negative	Positive	Positive
6	1	Positive	Invalid	Negative	Positive

[a] Specimens are grouped based on shared test results (indicated in columns 3 to 6).
[b] The results for both assays were identical.

the Illumigene *C. difficile* assay, 2 of which were 027/NAP1/BI strains (Table 2). The NAAT and CCNA showed a specificity of 100%. The Xpert *C. difficile* assays and the CCNA showed the highest sensitivity (100%) compared to Illumigene *C. difficile* (94%). Of the three discrepant specimens, all were positive by toxigenic culture, and two were also positive for 027/NAP1/BI (Table 2). Of the 43 prospective *tcdB*-positive specimens by the Xpert *C. difficile* assays, the Xpert *C. difficile*/Epi assay reported 2 (5%) as positive for *tcdB* and *cdt*, 32 (74%) as positive for *tcdB* alone, and 9 (21%) as positive for *tcdB*, *tcdC* deletion, and *cdt*. The latter were reported as presumptive 027/NAP1/BI (Table 3). For the retrospective *C. difficile* positive stool specimens, the Xpert *C. difficile*/Epi assay reported 4 *C. difficile* as positive for *tcdB* and *cdt*, 19 as positive for *tcdB* alone, and 27 as 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).

DISCUSSION

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 *C. difficile* in stool specimens, followed by either CCNA testing, toxigenic culture, or NAAT to identify toxin-producing *C. difficile* isolates (4). Although 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 ca. 71 to 100% (9, 19, 20). In addition, the two-step approach can cause a delay of 48 to 92 h that would require contact the isolation for patients with suspected CDI until the testing is complete.

At this time, four 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 *C. difficile* assay uses loop-mediated isothermal amplification technology to detect *tcd*A in the pathogenicity locus of toxigenic *C. difficile*. The test includes a manual extraction step but does not require costly capital equipment, and results are available in ~1 h. The Xpert *C. difficile*, BD GeneOhm *C. difficile* (BD Diagnostics, La Jolla, CA), and proGastro *C. difficile* (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 of the enhanced sensitivity of the assays detecting the *tcdA* or the *tcdB* genes and not the actual toxins, the testing of *C. difficile* should be limited to patients with clinical symptoms of CDI (6).

In the present study, we compared the sensitivity and specific-

TABLE 2 Discrepant analysis of stool specimens between Xpert *C. difficile*, Xpert *C. difficile*/Epi, and Illumigene *C. difficile* assays with toxigenic culture

Specimen[a]	Test result				
	Xpert *C. difficile* and Xpert *C. difficile*/Epi	Illumigene *C. difficile*	CCNA	Toxigenic culture	Presumptive 027/NAP1/BI[b]
P1	Positive	Negative	Negative	Positive	Negative
P2	Positive	Invalid	Negative	Positive	Positive*
P3	Positive	Negative	Positive	Positive	Positive*
P4	Positive	Negative	Negative	Positive	Negative
P5	Positive	Negative	Positive	Positive	Negative
P6	Positive	Negative	Positive	Positive	Negative
P7	Positive	Negative	Negative	Positive	Negative
P8	Positive	Negative	Negative	Positive	Negative
R1	Positive	Negative	Positive	Positive	Negative
R2	Positive	Negative	Positive	Positive	Positive*
R3	Positive	Negative	Positive	Positive	Positive*

[a] P, prospective stool specimens; R, retrospective stool specimens.
[b] *, 027/NAP1/BI results were confirmed by PFGE and *tcdC* sequencing.

TABLE 3 Presumptive identification of 027/NAP1/BI strain by the Xpert *C. difficile*/Epi assay in toxin B-positive stool specimens

No. of prospective specimens	No. of retrospective specimens	Presence (+) or absence (−)		
		tcdB	*tcdC* deletion	*cdt*
2	4	+	−	+
32	19	+	−	−
9 (027/NAP1/BI)	27 (027/NAP1/BI)	+	+	+

ity of the CCNA to the Xpert *C. difficile* assays and the Illumigene *C. difficile* assay. Although both the Xpert *C. difficile* and the Illumigene *C. difficile* assays showed greater sensitivity and quicker TATs (45 min and 1 h, respectively) compared to the CCNA (the median TAT for the positive specimens was 24 h [range, 6 to 72 h]), the Xpert *C. difficile* assays were more sensitive than the Illumigene *C. difficile* assay. In the prospective arm of the study, 10.5% specimens were positive overall by the CCNA compared to 17.5 and 21.5% by the Illumigene *C. difficile* and Xpert *C. difficile* assays, respectively (Table 1). The 027/NAP1/BI prevalence was 21%. The toxigenic culture of discrepant specimens showed the Xpert *C. difficile* assays to have detected 24 (100%) and the Illumigene *C. difficile* assay to have detected 17 (71%) of 24 true positives. Overall, the Xpert *C. difficile* assays detected an eight additional *C. difficile*-positive specimens, three of which were also CCNA positive (Table 1).

In addition to the *tcdB*, the Xpert *C. difficile*/Epi presumptively identifies the 027/NAP1/BI strain by detecting the binary toxin gene, and the *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 *C. difficile*/Epi were confirmed by PFGE (25) and/or sequencing of the *tcdC* gene (22). All nine strains identified were positive for all three markers (Table 3). The agreement between these methods was 100% (data not shown). One 027/NAP1/BI-positive specimen was determined to be invalid by the Illumigene *C. difficile* 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 also positive by the Xpert *C. difficile* assays, whereas only 47 of 50 specimens were determined to be positive by the Illumigene *C. difficile* assay (Table 2). Of the three specimens not detected by the Illumigene *C. difficile* assay, two were 027/NAP1/BI positive (Table 2). Although the reason for the low sensitivity of the Illumigene *C. difficile* assay is not clear, we speculate that organism load, mutations, or polymorphisms in primer- or probe-binding regions may affect detection of *C. difficile tcdA* variants, resulting in false-negative results.

The lack of detection of the *tcdA* in 027/NAP1/BI-positive strains by the Illumigene *C. difficile* assay is a concern. The 027/NAP1/BI strain is responsible for widespread outbreaks of *C. difficile* 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 as an epidemiological marker is known, and outbreaks caused by a toxin variant epidemic strain have renewed interest in detecting this strain. With regard 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 health care setting (13). These strains are associated with both community-acquired and health care-associated CDI. However, a preliminary review of patients with or 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 *C. difficile* assays had the highest sensitivity of the assays investigated in here; the assay detected all potential positive results, as confirmed by toxigenic culture. Perhaps most importantly for the accurate diagnosis of this infectious disease is the fact that the rapid, real-time PCR assay had a sensitivity similar to that of culture for detecting toxigenic *C. difficile*, while retaining the specificity of the direct cytotoxicity test. In a study by Novak-Weekly et al. (18), Xpert *C. difficile* testing yielded the highest sensitivity and negative predictive value in the least amount of time of the individual- and multiple-test algorithms evaluated.

The reagent cost for each assay and the amount of technical time required to perform it were as follows: $46 and 4 min, respectively, for the Xpert *C. difficile* assay; $26 and 5 min, respectively, for the Illumigene *C. difficile* assay; $12 and 5 min, respectively, for CCNA; and $27 and 30 min, respectively, for anaerobic culture. Despite the higher cost, the greatest impact of adopting the Xpert *C. difficile* assays will be in effectively reducing the time patients are kept in isolation. The 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 the Illumigene *C. difficile* assay, the Xpert *C. difficile* assay, and CCNA. This approach, could potentially affect the overall sensitivity and specificity of the two assays tested.

In conclusion, the Xpert *C. difficile* assays were more sensitive for the detection of toxigenic *C. difficile* and for the laboratory confirmation of CDI compared to the Illumigene *C. difficile* assay.

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