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Evaluation of a Loop-Mediated Isothermal Amplification Assay for Diagnosis of *Clostridium difficile* Infections▽

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A new assay (*illumi*gene *C. difficile*; Meridian Bioscience), based on the original loop-mediated isothermal amplification (LAMP) assay, was evaluated with 472 unformed stools from patients suspected of *Clostridium difficile* infection. Compared to the toxigenic culture, the sensitivity, specificity, and positive and negative predictive values were 91.8, 99.1, 91.8, and 99.1% for the *illumi*gene *C. difficile* assay and 69.4, 100, 100, and 96.6% for the cytotoxicity assay, respectively.

Clostridium difficile is the major causative agent of healthcare-associated diarrhea and pseudomembranous colitis. *C. difficile* infections (CDIs) have increased in frequency and severity in North America and Europe over the last 5 years, largely due to the emergence and rapid spread of the 027/NAP1/BI strain (9, 12). The diagnosis of CDI is usually based on a clinical history of recent antimicrobial usage and diarrhea in combination with laboratory tests. Currently, the "gold standards" for the diagnosis of CDI are the stool cytotoxicity assay (CTA) and the toxigenic culture (TC) (4, 6, 7). The stool cytotoxicity assay is not standardized, and it requires skill and facilities for cell culture. The toxigenic culture is slow and laborious, often requires 48 to 72 h to complete, and therefore is unlikely to be adopted by a clinical laboratory as the standard method for *C. difficile* testing. As of today, most laboratories have adopted enzyme immunoassays (EIAs) for toxins A and B as the routine method of testing (2). These techniques are easier to perform, more rapid than the other assays, and do not require specific technical skills. However, they are not sensitive enough to be used as a stand-alone test for *C. difficile* diagnosis (4, 6, 8, 15). More recently, real-time PCR assays have been commercially developed in order to overcome the lack of sensitivity of EIAs and to reduce the time of culture. These assays are designed to detect conservative region of the toxin B gene (*tcdB*) within the locus of pathogenicity (PaLoc) (ProdGastro Cd [Prodesse], BD GeneOhm Cdiff [Becton Dickinson], and Xpert *C. difficile* [Cepheid]). They were cleared by the Food and Drug Administration (FDA) for U.S. laboratory use. In Europe, the Xpert *C. difficile* assay targets *tcdB* in combination with binary toxin and deletion of *tcdC* for the presumptive identification of the 027 clone. This assay does not have FDA clearance for distribution in the United State, whereas it is commercially available in Europe. Numerous clinical studies have shown that these assays exhibit the best concordance with the results of toxigenic culture compared to enzyme immunoassays and therefore could represent a promising alternative for the diagnosis of CDI (8, 10, 11, 14, 16–19).

We performed a prospective study to evaluate the performance of a new amplification assay (*illumi*gene *C. difficile*, Meridian Bioscience, Cincinnati, OH), based on the original loop-mediated isothermal amplification (LAMP) technology, which targets within the PaLoc a conserved 204-bp sequence in the 5′ portion of the toxin A gene (*tcdA*). This assay was released by the FDA in July 2010 for U.S. laboratory use. The *illumi*gene assay was compared to the cytotoxicity assay and to the toxigenic culture.

Consecutive diarrheal stools ($n = 472$) (stools taking the shape of the container) from patients suspected of having CDIs were included. Stools were stored at +4°C until processing and analysis had been done (within 24 to 72 h of collection). The three methods (*illumi*gene, CTA, and TC) were performed on the same day. The results of the *illumi*gene were not known by the technicians performing the CTA or TC (blind evaluation).

CTA was performed using MRC-5 cells. Fresh stool specimens were diluted in phosphate-buffered saline (PBS) buffer (1:10 [wt/vol]) and centrifuged at 2,500 × *g* for 30 min. The supernatant was passed through a 0.45-μm-pore-size filter and inoculated onto confluent monolayers of MRC-5 cells in 96-well microtiter plates that were incubated at 37°C in a 6.5% CO_2 atmosphere for 48 h. The final dilution of stools tested was 1:100. Samples were considered positive if a characteristic cytopathic effect (cell rounding) was observed for at least 50% of the cells and could be neutralized with anti-*Clostridium sordellii* antiserum (obtained from M. R. Popoff, National Reference Center for Anaerobes, Pasteur Institute, Paris, France).

Toxigenic culture was performed on selective medium TCCA (brain heart infusion [BHI] broth supplemented with 5% defibrinated horse blood, 0.1% taurocholate, 250 μg/ml cycloserine, and 10 μg/ml cefoxitin), and plates were incubated for 48 h at 37°C in an anaerobic atmosphere. Colonies were identified by use of an enzymatic profile from the RapID32A gallery (bioMérieux, La Balme les Grottes, France). Then

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TABLE 1. Performance of the cytotoxicity assay and *illumi*gene *C. difficile* assay for detection of toxigenic strains of *C. difficile*

Assay result	No. of toxigenic culture results:		Assay performance (95% confidence interval)				
	Negative	Positive	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Correlation (%)
Cytotoxicity assay							
Negative	423	15	69.4 (56.5–82.3)	100 (94.9–98.3)	100	96.6 (94.9–98.3)	96.8
Positive	0	34					
Total	423	49					
*illumi*gene							
Negative	419	4	91.8 (84.2–99.5)	99.1 (98.1–99.9)	91.8 (84.2–99.5)	99.1 (98.1–99.9)	98.3
Positive	4	45					
Total	423	49					

C. difficile isolates were incubated in BHI broth for 5 days, and the supernatant was tested using the cytotoxicity assay.

The *illumi*gene *C. difficile* assay was performed according to the manufacturer's procedure. A specific collection brush was dipped into diarrheal stool samples and what was collected was added to a sample preparation chamber containing dilution buffer and formalin-inactivated-*Staphylococcus aureus* as an internal control of sample preparation and amplification. Genomic DNA was extracted by being heated at 95°C for 10 min. Under isothermal conditions at 65°C, amplification of the *tcdA* gene and *S. aureus* gene within two separated chambers was accomplished by the use of specially designed primers. Magnesium pyrophosphate was produced as a result of the amplification, which causes the reaction solution to become turbid. This turbidity was measured after 40 min using the *illumipro-10* incubator/reader (Meridian Bioscience, Cincinnati, OH). External quality controls, including a positive control (reconstituted DNA from the manufacturer's kit) and a negative control have been performed for each new kit lot. An invalid result caused by inhibition of amplification, incorrect sample preparation, or instrument or internal control failure led to repeat preparation of the sample and test according to the manufacturer's instructions.

The results from the *illumi*gene and CTA were compared to those from TC, considered the "gold standard" for CDI. In case of discrepant results between *illumi*gene and TC, additional testing was done. Stools that were *illumi*gene positive and TC negative were thawed and analyzed using an enrichment culture. Briefly, stools were inoculated into brain heart infusion broth containing prereduced taurocholate and cycloserine-cefoxitin (TCC broth) and incubated for 48 h at 37°C in an anaerobic atmosphere. Then, 100 μl of the broth was inoculated into a second TCC broth, which was incubated for 48 h in anaerobic atmosphere. The latter broth was plated on TCCA agar. Stools that were *illumi*gene negative and TC positive were tested again by *illumi*gene assay, using a new DNA extract. Moreover, the *illumi*gene assay was also performed with DNA extracted from the corresponding isolates.

Four samples gave an "invalid" result with the *illumi*gene assay (0.8%), which turned out to be positive for one specimen and negative for the others after repeat testing. These final results were used for interpretation. Of the 472 stool specimens, 49 (10.4%) were positive using the TC (gold standard). Of these, 34 were also positive using the CTA, and 45 were positive using the *illumi*gene. Compared to the TC, the sensitivity, specificity, and positive and negative predictive values (PPV and NPV, respectively) were 69.4, 100, 100, and 96.8% for CTA and 91.8, 99.1, 91.8, and 99.1% for the *illumi*gene *C. difficile* assay (Table 1).

Discordant results between the three methods and additional testing results are summarized in Table 2. No CTA-positive and *illumi*gene-negative results were obtained. Four specimens *illumi*gene negative and TC positive were tested again with *illumi*gene after a new extraction. One of these became positive, whereas the three others remained negative. Furthermore, the four isolates gave positive results using *illumi*gene amplification. Among the four specimens that were *illumi*gene positive but TC negative, three appeared to be true positive by enriched toxigenic culture.

After retesting of stools with discrepant results, the corrected sensitivity and specificity of the *illumi*gene therefore would be 94.2% and 99.8%, respectively, compared to en-

TABLE 2. Analysis of discrepant results between *illumi*gene *C. difficile* assay, cytotoxicity assay, and toxigenic culture

No. of specimens	Result from[a]:						
	Initial testing			Additional testing			
	Toxigenic culture	Cytotoxicity assay	*illumi*gene	*illumi*gene on new DNA extract	*illumi*gene on isolate	Enriched toxigenic culture	Final interpretation of *illumi*gene
1	Pos	Neg	Neg	Pos	Pos	ND	TP
3	Pos	Neg	Neg	Neg	Pos	ND	FN
3	Neg	Neg	Pos	ND	ND	Pos	TP
1	Neg	Neg	Pos	ND	ND	Neg	FP
11	Pos	Neg	Pos	ND	ND	ND	TP
0	Pos	Pos	Neg				

[a] Pos, positive; Neg, negative; ND, not done; TP, true positive; FN, false negative; FP, false positive.

riched toxigenic culture. The reasons why direct toxigenic culture appeared negative could include a low concentration of microorganisms in a very heterogeneous sample or growth inhibition due to previous therapy for *C. difficile*.

Our results fully confirm the recent findings of Noren et al., who reported a sensitivity and specificity of the *illumi*gene of 98% (13). This technology is innovative, and the target (*tcdA*) is different from those of other real-time PCR-based methods, which mostly focus on the *tcdB* gene. Variability in genes coding for toxins is not a rare phenomenon. Rupnik et al. have shown that 21.5% of *C. difficile* strains are variant for the toxin A and B genes (16) and that *tcd*A was more conserved than *tcdB*. One potential concern regards the detection of A^- B^+ variant strains. Those strains belong to toxinotype VIII or X. They represent 3.9% of *C. difficile* isolates in a recent pan-European survey of *C. difficile* infection (3). Actually, the *illumi*gene *C. difficile* assay detects the PaLoc by targeting a DNA fragment in the 5′ region of the *tcdA*, which is intact in all strains, including those with a large deletion in the *tcdA* gene. Coyle et al. recently showed that *illumi*gene *C. difficile* was positive in stools spiked with A^- B^+ strains from toxinotypes VIII and X (5). Many clinical trials have recently evaluated the performance of the real-time PCR-based methods currently available on the market. Their sensitivity and specificity range from 77.3% to 97.1% and 93% to 100%, respectively (1, 8, 10, 11, 14, 16–19). The performance characteristics of these *illumi*gene *C. difficile* assays are in agreement with those data, with sensitivity and specificity of 91.8% and 99.1%, respectively. For a batch of 10 stool samples, the "hands on" time is 20 min, and the overall turnaround time is 1 h. Moreover the technology is isothermal and therefore requires no costly capital equipment.

In conclusion, the *illumi*gene *C. difficile* assay is the first FDA-approved isothermal nucleic acid amplification-based assay. It offers sensitivity and specificity for detection of toxigenic strains that are comparable to those of the toxigenic culture reference method and other PCR-based methods. Results are available within 1 h.

The combination of a quick turnaround time with high performance might result in better management of CDIs and timely implementation of infection control measures.

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