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Comparison of Illumigene, Simplexa, and AmpliVue *Clostridium difficile* Molecular Assays for Diagnosis of *C. difficile* Infection

E. Deak, S. A. Miller, R. M. Humphries

Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, Los Angeles, California, USA

We compared the performance of the Simplexa Universal Direct (Focus Diagnostics) and AmpliVue (Quidel Corporation) assays to that of the Illumigene assay (Meridian Bioscience, Inc.) for the diagnosis of *Clostridium difficile* infection. Two hundred deidentified remnant diarrheal stool specimens were tested by the Simplexa, AmpliVue, and Illumigene methods. Specimens with discrepant results among the three assays and a representative number of concordant specimens were further evaluated by toxigenic culture. The sensitivity and specificity were 98 and 100% and 96 and 100% for the Simplexa Universal Direct and AmpliVue assays, respectively. Both assays are easy to perform, with rapid turn-around-times, supporting their utility in the clinical laboratory as routine diagnostic platforms.

The management and control of *Clostridium difficile* infection (CDI) continue to present a formidable challenge in the 21st century for hospitals, long-term-care facilities, and nursing homes (1). Infection rates have increased markedly in the United States over the past decade (2–4), and health care costs associated with CDI are a substantial burden to the health care system (1). The availability of a rapid and accurate laboratory diagnostic test for CDI is essential for patient treatment and prevention of transmission (5). Nucleic acid amplification tests (NAATs) that target the toxin A and/or B genes of *C. difficile* have gained popularity among laboratories in the United States. These tests have better sensitivity than traditional toxin antigen-based assays and culture (6) and have been shown to be a cost-effective alternative to traditional diagnostic methods (7). In recent years, a plethora of *C. difficile*-specific NAATs have been approved or cleared by the U.S. Food and Drug Administration (FDA). This study evaluated the performance of two newly cleared *C. difficile* assays, the Simplexa Universal Direct and AmpliVue *C. difficile* assays, in comparison with that of the Meridian Illumigene assay and toxigenic *C. difficile* culture. The performance characteristics of the Illumigene assay compared to the "gold standard" toxigenic culture (TC) in detecting toxigenic *C. difficile* in clinical stool samples have been established previously. Lalande et al. showed that Illumigene had higher sensitivity (91.8% compared to TC) than the cytotoxicity assay (69.4%) when they looked at 476 stool specimens (8). Studies by Norén et al. showed 98% sensitivity and specificity when they compared Illumigene with TC in their study of 272 consecutive stool samples (9). More recently, studies evaluating multiple molecular platforms against TC as the reference method have shown sensitivities of 86.7 to 93.3% (10, 11). Overall, the Illumigene assay has proven to be as reliable as TC in detecting infection by toxigenic *C. difficile* in routine clinical settings.

The Simplexa *C. difficile* Universal Direct real-time PCR assay uses bifunctional fluorescent probes-primers to amplify a conserved region of the toxin B gene (*tcdB*) in *C. difficile* directly in heat-treated stool samples (12). The AmpliVue *C. difficile* assay uses helicase-dependent amplification technology for isothermal amplification of a highly conserved 83-bp fragment of the 5′ end of the toxin A gene (*tcdA*) and a self-contained disposable amplification detection device that incorporates a lateral-flow strip for visual evaluation of assay results (13). The Illumigene *C. difficile* assay uses loop-mediated isothermal DNA amplification (LAMP) technology to target a partial DNA fragment of *tcdA* (14).

Fresh, unformed stool samples submitted to the laboratory for *C. difficile* detection between January and March 2013 were tested by the Illumigene *C. difficile* assay in accordance with the manufacturer's instructions. For each positive specimen, two negative specimens were randomly selected daily, for a total of 50 positive and 150 negative specimens. Duplicate specimens from the same patients were excluded. These remnant stool specimens were assigned study numbers, deidentified, and tested by the Simplexa *C. difficile* Universal Direct assay and the AmpliVue *C. difficile* assay on the same day as Illumigene testing was performed. Two of three specimens with discordant results among the three assays ($n = 2$), along with an equal number of concordant specimens ($n = 2$), were sent to a reference laboratory for further testing by TC (15, 16). One specimen with discrepant results could not be sent because of insufficient volume. This study was approved by the local Institutional Review Board.

All NAATs were performed according to the manufacturer's specifications. Briefly, for the Simplexa *C. difficile* Universal Direct assay, a flocked swab was dipped into a thoroughly mixed stool specimen, transferred into Tris-EDTA (TE) buffer, and heated at 97°C for 10 min (swab and TE buffer not provided by the manufacturer). A reaction mixture was prepared and added to each sample and control well of a 96-well Universal Disc for the Simplexa *C. difficile* Universal Direct assay, followed by the addition of a heat-treated patient sample, an unheated positive control, or a heat-treated no-template control (TE buffer). The disc was then loaded into the Integrated Cycler. Results were recorded as positive (C_T value of <40 with or without a valid internal control curve), negative (C_T value of 0 or ≥ 40 with a valid internal control

Received 28 August 2013 **Returned for modification** 25 September 2013
Accepted 11 December 2013
Published ahead of print 18 December 2013
Editor: R. Patel
Address correspondence to Eszter Deak, EDeak@mednet.ucla.edu.

doi:10.1128/JCM.02354-13

TABLE 1 Performance of the Focus Simplexa Universal Direct and Quidel AmpliVue assays compared to that of the Meridian Illumigene assay

Assay and result	No. of Meridian Illumigene assay results:		% Sensitivity (95% CI)	% Specificity (95% CI)
	Positive	Negative		
Focus Simplexa Universal Direct				
Positive	49	1[b]	98 (88–99.9)	100 (96.9–100)
Negative	1[a]	149		
Quidel AmpliVue				
Positive	48	0	96 (85.1–99.3)	100 (96.9–100)
Negative	2	150		

[a] Positive by TC.
[b] Sample disregarded because of insufficient specimen amount, inability to confirm by TC.

curve), or invalid (C_T value of 0 with an invalid control curve). For the AmpliVue *C. difficile* assay, stool samples were collected with the sterile swab provided by the manufacturer and transferred into the dilution buffer tube, and 50 μl of the diluted stool specimen was transferred to lysis buffer. The sample was then heated at 95°C for 10 min, and 50 μl of the lysed sample was transferred to a reaction tube. The reaction tube was incubated at 64°C for 60 min, placed into the amplicon cartridge, inserted into the detection cassette, and read after 10 min. The results were recorded as positive (visible red line at T2 with or without a visible red C line), negative (visible red C line only), or invalid (no visible red lines). For the Illumigene *C. difficile* LAMP assay, stool samples were collected on the manufacturer-provided sample brush and transferred to sample diluent. The sample was vortexed, and 5 to 10 drops were squeezed into an Illumigene extraction tube. The tube was heated at 95°C for 10 min and vortexed, and 50 μl of the extracted sample was transferred to an Illumigene reaction buffer tube. The reaction buffer tube was vortexed, and 50 μl was transferred to the test chamber and control chamber of the Illumigene assay device containing the appropriate beads. The Illumigene device was then inserted into an Illumipro-10 for amplification and detection. The results were recorded as positive, negative, or invalid. Two of three discordant specimens were further tested by enhanced TC. A portion of the specimen was cultured for 2 days in a prereduced cooked meat broth (CMG). The broth was subcultured onto plates of prereduced cefoxitin-cycloserine fructose agar modified with horse blood. After 48 h of incubation, colonies resembling *C. difficile* were identified by Gram staining, production of proline aminopeptidase, vancomycin susceptibility, and aerotolerance. Isolates of *C. difficile* were tested for the production of toxin B by testing the CMG culture with the same cytotoxic assay kit used to test the original specimen. Following the study period, patients were evaluated by a retrospective chart review to document disease severity and antibiotic therapy at the time of specimen collection. Patients were classified as having severe CDI if they had either endoscopic evidence of pseudomembranes or treatment in an intensive care unit or if they had two of the following risk factors: an age of >60 years, a temperature of >38.3°C, an albumin level of <2.5 mg/dl, or a white blood cell count of >15,000 cells/mm^3 within 48 h of specimen collection for *C. difficile* testing as defined by Zar and colleagues (17).

Of 200 specimens tested, 50 were positive and 150 were negative by the Simplexa *C. difficile* Universal Direct assay. Two specimens were discrepant with respect to the Illumigene assay, one false positive and one false negative (Table 1). The false-negative specimen was also falsely negative by the AmpliVue *C. difficile* assay with Illumigene as the reference but was positive by TC (Table 2). The false positive was not tested by TC because of limited specimen availability but was negative by the AmpliVue *C. difficile* assay. This specimen had a relatively high cycle threshold (C_T) value of 38.3 and was loaded next to a sample with a lower C_T value (30.3); it is therefore possible that the false positivity was due to cross-contamination during sample loading, although this was not resolved at the time of the discrepancy. Meanwhile, 48 samples tested positive and 152 tested negative by the AmpliVue *C. difficile* assay (Table 1). Two specimens were discrepant with the Illumigene assay. In addition to the false negative shared by the Simplexa *C. difficile* Universal Direct assay, a second false negative occurred that was positive by both the Simplexa *C. difficile* Universal Direct assay and TC. Both concordant specimens were confirmed by TC. Overall, the Simplexa *C. difficile* Universal Direct assay and the AmpliVue *C. difficile* assay showed 98.7% concordance with the Illumigene assay. The Simplexa *C. difficile* real-time PCR assay was 98% (95% confidence interval [CI], 88 to 99.9%) sensitive and 100% (95% CI, 96.9 to 100%) specific, and the AmpliVue *C. difficile* assay was 96% (CI, 95%, 85.1 to 99.3%) sensitive and 100% (CI, 95%, 96.9 to 100%) specific in comparison with the Illumigene *C. difficile* assay. Predictive values were not calculated, as sampling was not representative of the true prevalence in the population. TC yielded results that were consistent with results obtained by the Illumigene assay. Of the patients who were positive for *C. difficile* by the Illumigene assay, 16 had severe disease and 34 had mild disease. Discordant specimens were from patients with mild disease.

TABLE 2 Method comparison for 10 samples

Parameter	Illumigene	AmpliVue	Simplexa
Throughput[a]	1–10	1–24	1–94
Specimen preparation time (min)	10	3	3
Lysis time (min)	10	10	10
Reaction preparation time (min)	8	5	5
Amplification time (min)	40	60	55
No. of steps postamplification	0	1	0
Time postamplification (min)	0	13	0
Total time to completion (min)	68	91	73
Hands-on time (min)	18	11	8

[a] Throughput is the total possible number of samples per run.

The three assays were compared in a timed run of one operator processing 10 samples. Throughput, number of steps, amount of time required for the individual steps, total hands-on time, and time to completion were evaluated (Table 2). The Simplexa *C. difficile* Universal Direct assay can accommodate a higher capacity of patient specimens per instrument ($n = 1$ to 94) than the Illumigene ($n = 1$ to 10) and AmpliVue ($n = 1$ to 24) platforms can. The total assay time was the shortest for the Illumigene assay, 68 min versus 91 and 73 min for the Simplexa and AmpliVue assays, respectively. The hands-on time per batch of 10 samples was shorter for both the Simplexa (8 min) and AmpliVue (11 min) assays than for Illumigene (18 min). The AmpliVue assay requires an additional step in which the amplification product is transferred to a disposable cartridge containing a lateral-flow strip and incubated for 10 min prior to the final readout.

The continuously expanding market of FDA-cleared NAATs reflects the need for rapid and accurate diagnostic tests for CDI. There are currently several commercially available FDA-cleared NAATs that are highly sensitive and specific for the detection of toxigenic *C. difficile* directly in stool specimens, all within 2 h. These include the BD Gene-Ohm, Roche LightCycler, Cepheid Xpert, Gen-Probe ProGastro, Verigene Nucleic Acid, Great Basin Scientific Portrait Toxigenic, and Meridian Illumigene *C. difficile* assays, most of which have been evaluated in the literature (18). In the present study, we compared the sensitivity and specificity of the Simplexa Universal Direct and AmpliVue *C. difficile* assays to those of the Illumigene *C. difficile* assay. The sensitivity and specificity of both methods were similar to those reported for other test systems (8, 9, 11, 19–21). The limitations of this study include the selection of positive specimens based on Illumigene results and the use of the Illumigene assay as the reference method. Additionally, the performance of TC was limited to discrepant results. Parameters such as ease of use, hands-on time, time to completion, and flexibility of the platform for the detection of other organisms vary among the available NAAT assays and can have a significant impact on defining which test will integrate best into the existing workflow and ultimately its suitability for a given clinical microbiology laboratory, given the institutions and patient populations served by that laboratory. The three FDA-cleared assays compared in this study were easy to perform, involving few steps and minimal hands-on time, with simple specimen and reaction preparation, and had rapid turnaround times. The Simplexa assay may be an advantage in labs with higher test volumes because of the lower number of steps and minimal hands-on time. Additionally, an entire disc is consumed whether an individual sample or a batch of samples is run, making it more appropriate for high-volume laboratories. Furthermore, the Simplexa platform, a Focus 3M Integrated Cycler, can be used for other assays offered by Focus Diagnostics, which may make the Simplexa *C. difficile* assay more appealing to laboratories already using this platform. The AmpliVue assay, with its individual cartridges, allows individual tests to be run without incurred waste of materials and may be well suited for low- to mid-volume labs, being able to accommodate up to 24 samples at a time with the provided heat blocks. In conclusion, the Simplexa and AmpliVue *C. difficile* assays are rapid and sensitive methods for the detection of *C. difficile* in clinical stool specimens, suggesting a role in the clinical laboratory as routine diagnostic tests for CDI.

ACKNOWLEDGMENTS

We are grateful to Quidel Corporation and Focus Diagnostics for providing reagents for this study. We thank Ron Lollar, for performing the TC assays.

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