Molecular Test Based on Isothermal Helicase-Dependent Amplification for Detection of the *Clostridium difficile* Toxin A Gene

Catherine Eckert,^{a,c} Eleonore Holscher,^a Amandine Petit,^{a,c} Valérie Lalande,^{a,b,c} Frédéric Barbut^{a,b,c}

Assistance Publique-Hôpitaux de Paris, Hôpital Saint-Antoine, National Reference Laboratory for *C. difficile*, Paris, France^a; Assistance Publique-Hôpitaux de Paris, Hôpital Saint-Antoine, Microbiology Unit, Paris, France^b; UPMC, Université Paris 06, GRC-2, Epidiff, Paris, France^c

The AmpliVue *Clostridium difficile* assay and a glutamate dehydrogenase (GDH)-*illumigene* algorithm were evaluated using 308 diarrheal stool specimens of patients suspected of having *C. difficile* infection. Compared to the enriched toxigenic culture method, the sensitivities, specificities, and positive and negative predictive values of the AmpliVue *C. difficile* assay and the GDH-*illumigene*-based algorithm were 91.7% (95% confidence interval [CI], 76.4 to 97.8), 100% (95% CI, 98.3 to 100), 100% (95% CI, 87 to 100), and 98.9% (95% CI, 96.6 to 99.7), respectively.

Toxigenic *Clostridium difficile* strains are the leading cause of health care-associated diarrhea among adults from industrialized countries (1). To avoid dissemination of this pathogen, its prompt and reliable diagnosis is essential. The toxigenic culture (TC) and the stool cytotoxicity assay (CTA) are still considered the reference methods, but they are both time-consuming (2). The stool CTA consists of inoculating a stool filtrate on cell culture and observing a specific cytopathic effect (rounding of the cells) after 24 to 48 h of incubation. The CTA detects the presence of free *C. difficile* toxins in a stool sample (mainly toxin B) and is very specific for the disease. TC is a 2-step method. First, *C. difficile* is isolated on a selective medium, and then the *in vitro* toxigenicity of the strain is determined. It is a sensitive method that detects the presence of *C. difficile* strains that have the potential to produce toxins A and B. Enzyme immunoassays (EIA) for toxins A and B, although easy to perform and widely used, are not sensitive enough to be used as stand-alone tests (3, 4). European and American guidelines now propose implementation of the EIA for detecting glutamate dehydrogenase (GDH), a specific enzyme of *C. difficile*, as a screening method (3, 5, 6). These tests display high negative predictive values (NPVs), allowing the diagnosis of *C. difficile* infection (CDI) to be ruled out in the case of a negative result; however, a positive result should be confirmed by a second specific test for toxin detection (7). Nucleic acid amplification tests (NAATs) are the most recent tests developed for the diagnosis of CDI. They detect genes coding for toxin A (*tcdA*) and/or B (*tcdB*). Some tests, such as the Xpert *C. difficile* (Cepheid) or the BD GeneOhm Cdiff assay (BD Diagnostics), are based on real-time PCR, and other tests, such as the *illumigene* assay (Meridian Bioscience) and the AmpliVue *C. difficile* assay (Quidel), use isothermal amplification. NAATs are very sensitive compared to TC (8). However, a positive result should be interpreted with caution because these methods can detect “asymptomatic” patients who carry a toxigenic strain and who have diarrhea for other reasons.

The AmpliVue *C. difficile* assay (Quidel) is a new molecular test based on isothermal helicase-dependent amplification (HDA), and it has a readout like that of a traditional lateral flow assay. It targets the highly conserved region of *tcdA* so that A⁺ B⁺ strains (which retain part of the conserved *tcdA*) can be detected. The objectives of this study were to evaluate the performances of the AmpliVue *C. difficile* assay and a two-step algorithm, based on GDH detection (Quik Chek; Alere) followed by *illumigene* (Me-

ridian Bioscience) testing on positive GDH samples, in comparison to the CTA and TC.

MATERIALS AND METHODS

This prospective study was conducted at the National Reference Laboratory for *Clostridium difficile* in Paris, France. A total of 308 consecutive diarrheal stool samples (defined as stool samples taking the shape of the container) was collected from November 2012 to April 2013 from patients (≥ 2 years old) suspected of having CDI and hospitalized in one of four different university-affiliated hospitals in Paris (Saint-Antoine, Tenon, Trousseau, and Rothschild hospitals). Only diarrheic stool samples were included. *C. difficile* testing was done in the case of a specific request from the physician and systematically in all cases of nosocomial diarrhea (occurring after day 3 of hospitalization). The stool samples were stored at 4°C and tested within 3 days after sample collection. An aliquot was stored at -80°C for further testing in case of discordant results. CTA were performed using MRC-5 cells as described elsewhere (9). Cultures were performed on the selective medium TCCA (brain heart infusion [BHI] agar supplemented with 5% defibrinated horse blood, 0.1% taurocholate, 250 $\mu\text{g}\cdot\text{ml}^{-1}$ cycloserine, and 8 $\mu\text{g}\cdot\text{ml}^{-1}$ cefoxitin, homemade) (10). *In vitro* determinations of the cytotoxicity of *C. difficile* isolates were performed by inoculating 2 to 5 colonies into BHI broth that was incubated for 5 days in an anaerobic atmosphere. The supernatant from this culture was filtered and inoculated on MRC-5 cells (TC). The Quik Chek (Alere), *illumigene* (Meridian Bioscience), and AmpliVue (Quidel) assays were performed directly from stool samples according to the manufacturers' instructions. The *illumigene* assay was performed in cases of positive GDH results to determine whether a strain was toxigenic. Invalid results with the AmpliVue assay were defined as results that were not validated because of the absence of a visible band in the control and test lines. In the case of discordant results (defined as results of the AmpliVue assay or the GDH-*illumigene* algorithm that did not match with the results of TC), the samples that were stored at -80°C were tested again by the same methods and/or by enriched TC. For the enriched TC, stool samples were inoculated in prerduced taurocholate-cycloserine-cefoxitin BHI broths that

Received 7 February 2014 Returned for modification 11 March 2014

Accepted 15 April 2014

Published ahead of print 23 April 2014

Editor: E. Munson

Address correspondence to Catherine Eckert, catherine.eckert@sat.aphp.fr.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JCM.00353-14

TABLE 1 Results of all of the methods before (first test) and after (second test) resolving discrepant results

No. with results (<i>n</i> = 308)	First test result for:					Second test result for:			
	Cytotoxicity assay	Toxigenic culture	GDH	<i>illumigene</i>	AmpliVue	Enriched toxigenic culture	GDH	<i>illumigene</i>	AmpliVue
253	—	—	—	ND ^a	—	ND	ND	ND	ND
22	+	+	+	+	+	ND	ND	ND	ND
8	—	+	+	+	+	ND	ND	ND	ND
11	—	—	+	—	—	ND	ND	ND	ND
1	—	+	+	+	—	ND	ND	ND	+
1	+	+	+	—	—	ND	ND	+	+
2	—	+	+	—	—	+	ND	—	—
1	—	+	+	—	+	ND	ND	+	ND
1	—	+	—	ND	—	+	—	—	—
1	—	—	+	+	—	—	ND	—	—
1	—	—	+	—	+	—	ND	ND	—
5	—	—	—	ND	+	—	—	ND	—
1	—	—	—	ND	Inv ^b	ND	ND	ND	Inv

^a ND, not done.^b Inv, invalid.

were incubated for 5 days at 37°C under anaerobic conditions, and then the broths were plated onto TCCA agar. The capacity of the strain to produce toxins *in vitro* was determined after DNA extraction from colonies by in-house PCR targeting *tcdA* and *tcdB* (11).

In cases of positive TC results and negative AmpliVue assay results, the molecular method was performed directly on colonies to rule out a potential genetic drift of the *tcdA* gene. Briefly, 3 to 5 colonies were picked up and suspended into dilution buffer. The assay was then performed as described by the manufacturer.

RESULTS

Among the 308 stool samples processed, 23 (7.5%), 48 (15.6%), and 36 (11.7%) were positive by the stool CTA, by culture, and by TC, respectively. Four results (1.3%) were invalid with the AmpliVue assay because no band was visible for both control and test lines. After repeating the test, 1 result remained invalid (0.3%), and 3 were negative.

The total turnaround time for the AmpliVue assay was approximately 90 min versus 30 min for the GDH-*illumigene* algorithm in cases of a negative GDH result or 90 min when the 2 tests were performed. The total hands-on times for the AmpliVue and *illumigene* assays were <10 min each.

Compared to the CTA, the sensitivities, specificities, positive predictive values (PPVs), and NPVs for the AmpliVue assay were 95.7% (95% confidence interval [CI], 76 to 99.8), 94.7% (95% CI, 91.3 to 96.9), 59.5% (95% CI, 42.2 to 74.8), and 99.6% (95% CI, 97.6 to 100) and for the GDH-*illumigene* algorithm were 95.7% (95% CI, 76 to 99.8), 96.5% (95% CI, 93.4 to 98.2), 68.7% (95% CI, 49.9 to 83.3), and 99.6% (95% CI, 97.7 to 100), respectively. Compared to the TC, the sensitivities, specificities, PPVs and NPVs for the AmpliVue assay were 86.1% (95% CI, 69.7 to 94.8), 97.8% (95% CI, 95 to 99.1), 83.8% (95% CI, 67.3 to 93.2), and

98.1% (95% CI, 95.5 to 99.3) and for the GDH-*illumigene* algorithm were 86.1% (95% CI, 69.7 to 94.8), 99.6% (95% CI, 97.6 to 100), 96.9% (95% CI, 82 to 99.8), and 98.2% (95% CI, 95.6 to 99.3), respectively. The discrepant results are listed in Table 1. After resolving the discrepant results, the sensitivities, specificities, PPVs, and NPVs for both the AmpliVue assay and the GDH-*illumigene* algorithm were 91.7% (95% CI, 76.4 to 97.8), 100% (95% CI, 98.3 to 100), 100% (95% CI, 87 to 100), and 98.9% (95% CI, 96.6 to 99.7), respectively, compared to the enriched TC. Among the 36 toxigenic culture-positive stool samples, 33 were positive with the AmpliVue assay and with the GDH-*illumigene* algorithm after resolving the discrepant results. Even after repeating the test, three samples were positive with TC but were still negative with the AmpliVue assay and the GDH-*illumigene* algorithm (Table 2). For these 3 discordant samples, the AmpliVue assay was performed on colonies, and the results were positive. No false-positive results were observed with the GDH-*illumigene* algorithm or with the AmpliVue assays after resolving the discrepant results.

DISCUSSION

There are many molecular methods now available on the market for the detection of *C. difficile* toxin genes (12). Most assays use real-time PCR and target *tcdB*, but the isothermal amplification of a highly conserved region of *tcdA*, as with the AmpliVue and *illumigene* assays using HDA and loop-mediated isothermal amplification (LAMP) technologies, respectively, is also very promising (13). The AmpliVue assay is characterized by a self-contained disposable amplicon detection device. This assay was approved by the FDA in 2012 and is labeled by the Conformité Européenne In Vitro Diagnostics (CE-IVD). To our knowledge, this is the first

TABLE 2 Analysis of the 3 false-negative results after resolving discrepant results

No. of samples	Result for:					
	Cytotoxicity assay	Toxigenic culture	GDH Quik Chek	<i>illumigene</i>	AmpliVue	AmpliVue on colonies
2	Negative	Positive	Positive	Negative	Negative	Positive
1	Negative	Positive	Negative	Negative	Negative	Positive

study to compare the performance of the AmpliVue assay to TC and the CTA. A recent study comparing the performances of the Simplexa Universal Direct (Focus Diagnostics) and AmpliVue molecular assays to that of the *illumigene* assay was published by Deak et al. (14). In their study, including 200 selected diarrheal stool samples, only discrepant results were evaluated by TC; the sensitivity and specificity were 96% and 100% for the AmpliVue assay.

Our results showed a sensitivity and specificity of 91.7% and 100%, respectively, for the AmpliVue assay and the GDH-*illumigene* algorithm. The sensitivity of the AmpliVue assay is in agreement with that in a recent meta-analysis indicating that the pooled sensitivity of molecular methods was 92% compared to TC (8). These results are also in line with the sensitivity of 94.2% found for the *illumigene* assay as a stand-alone test, another method that detects the *tcdA* gene (15). The results of a previous study reported by Bamber et al., including 810 consecutive stool samples, were in complete accordance with our results for the algorithm assay. The authors reported a sensitivity of 91.6% for their GDH (Premier EIA)-*illumigene* algorithm (16). However, a lower sensitivity (68.3%) of the GDH-*illumigene* algorithm was reported by Walkty et al. (17). This lower sensitivity was due to the false-negative results of the screening and the molecular test. Among the 63 TC-positive samples, 11 were missed due to a negative GDH assay and 9 due to a negative *illumigene* assay. This difference in sensitivity may also be associated with the lack of repeated testing in cases of discrepant results in the study performed by Walkty et al. However, the sensitivity was still lower than the 86.1% sensitivity we found before the analysis of discordant results. The differences may then be related to the better sensitivity of their reference TC method based on alcohol shock, in-house selective medium, and PCR detecting *tcdA* and/or *tcdB* on colonies. Indeed, the study by Walkty et al. was one of the only studies to find such a low sensitivity for either the GDH or the molecular assay.

Three samples were TC positive, but the two molecular methods (AmpliVue and *illumigene*) gave negative results even after repeated testing (Table 2). To rule out a potential genetic drift of *tcdA*, the AmpliVue assay was performed directly on colonies for these 3 samples, and the results were positive. These results allowed the exclusion of a genetic drift in the target sequence, a potential limitation of molecular methods. These results may be explained by the presence of inhibitors in the stool samples, cross-contamination of the TC, or a lower sensitivity of detection for the molecular method.

Molecular methods are very sensitive and therefore very promising, but some limitations exist. The presence of inhibitors in stool samples or genetic drifts in the target sequence can lead to false-negative results. The emergence of strains that lack *tcdA*, such as PCR ribotype 237 strains isolated in piglets from Australia, or that partially lack *tcdA*, such as PCR ribotype 033 (toxintype XI) strains, would not be detected using NAATs that detect only *tcdA* (18, 19).

Generally, the interpretation of a positive result (with TC or the molecular method) in the absence of free toxins in the feces remains one subject of debate. In a study including >12,000 fecal samples, Planche et al. showed that the presence of free toxins best defines clinically relevant cases of CDI (20). A higher case fatality rate was observed among patients who were positive by the cell cytotoxicity assay compared to patients without free toxins but harboring a toxigenic strain. A positive result with a molecular

method cannot predict the presence of free toxins in the feces, and thus clinical assessment remains essential for identifying a true infection caused by *C. difficile*. It is absolutely necessary to accept only diarrheal stool samples that take the shape of the container for molecular testing. This will significantly reduce the possibility of detecting a potential *C. difficile* carrier.

In conclusion, the AmpliVue assay and the GDH-*illumigene* algorithm are both as rapid and sensitive as other molecular methods, but these two assays do not require costly equipment. Moreover, with the AmpliVue assay, the result and control of each sample are directly visualized on a strip, and it requires <10 min of hands-on time. The rapidity of the results with molecular methods is interesting for early recognition of excretors of toxigenic strains and rapid management of the patient, including isolation in a hospital setting. Nevertheless, when reporting results to the clinician, one should be mindful of the limits of these new methods, particularly their potential lack of specificity. In the case of a positive result, clinical assessment is essential.

ACKNOWLEDGMENTS

We are grateful to Quidel Molecular and Bühlmann France SAS for providing kits, funding, and technical support for this study.

REFERENCES

- McFarland LV, Mulligan ME, Kwok RY, Stamm WE. 1989. Nosocomial acquisition of *Clostridium difficile* infection. N. Engl. J. Med. 320:204–210. <http://dx.doi.org/10.1056/NEJM198901263200402>.
- Planche T, Wilcox M. 2011. Reference assays for *Clostridium difficile* infection: one or two gold standards? J. Clin. Pathol. 64:1–5. <http://dx.doi.org/10.1136/jcp.2010.080135>.
- Crobach MJ, Dekkers OM, Wilcox MH, Kuijper EJ. 2009. European Society of Clinical Microbiology and Infectious Diseases (ESCMID): data review and recommendations for diagnosing *Clostridium difficile* infection (CDI). Clin. Microbiol. Infect. 15:1053–1066. <http://dx.doi.org/10.1111/j.1469-0691.2009.03098.x>.
- René P, Frenette CP, Schiller I, Dendukuri N, Brassard P, Fenn S, Loo VG. 2012. Comparison of eight commercial enzyme immunoassays for the detection of *Clostridium difficile* from stool samples and effect of strain type. Diagn. Microbiol. Infect. Dis. 73:94–96. <http://dx.doi.org/10.1016/j.diagmicrobio.2012.01.005>.
- Cohen SH, Gerding DN, Johnson S, Kelly CP, Loo VG, McDonald LC, Pepin J, Wilcox MH. 2010. Clinical practice guidelines for *Clostridium difficile* infection in adults: 2010 update by the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA). Infect. Control. Hosp. Epidemiol. 31:431–455. <http://dx.doi.org/10.1086/651706>.
- American Society for Microbiology. 2010. A practical guidance document for the laboratory detection of toxigenic *Clostridium difficile*. American Society for Microbiology, Washington, DC.
- Shetty N, Wren MWD, Coen PG. 2011. The role of glutamate dehydrogenase for the detection of *Clostridium difficile* in faecal samples: a meta-analysis. J. Hosp. Infect. 77:1–6. <http://dx.doi.org/10.1016/j.jhin.2010.07.024>.
- O'Horo JC, Jones A, Sternke M, Harper C, Safdar N. 2012. Molecular techniques for diagnosis of *Clostridium difficile* infection: systematic review and meta-analysis. Mayo Clin. Proc. 87:643–651. <http://dx.doi.org/10.1016/j.mayocp.2012.02.024>.
- Chang TW, Gorbach SL, Bartlett JB. 1978. Neutralization of *Clostridium difficile* toxin by *Clostridium sordellii* antitoxins. Infect. Immun. 22:418–422.
- Barbut F, Giarizzo B, Bonne L, Lalande V, Burghoffer B, Luiuz R, Petit JC. 2007. Clinical features of *Clostridium difficile*-associated infections and molecular characterization of strains: results of a retrospective study, 2000–2004. Infect. Control. Hosp. Epidemiol. 28:131–139. <http://dx.doi.org/10.1086/511794>.
- Eckert C, Coignard B, Hebert M, Tarnaud C, Tessier C, Lemire A, Burghoffer B, Noel D, Barbut F, ICD-Raisin Working Group. 2013. Clinical and microbiological features of *Clostridium difficile* infections in

- France: the ICD-RAISIN 2009 national survey. *Med. Mal. Infect.* 43:67–74. <http://dx.doi.org/10.1016/j.medmal.2013.01.004>.
12. Barbut F, Lalande V, Eckert C. 2012. New molecular methods for the diagnosis of *Clostridium difficile* infections. *Drugs Today (Barc.)* 48:673–684.
 13. Brecher SM, Novak-Weekley SM, Nagy E. 2013. Laboratory diagnosis of *Clostridium difficile* infections: there is light at the end of the colon. *Clin. Infect. Dis.* 57:1175–1181. <http://dx.doi.org/10.1093/cid/cit424>.
 14. Deak E, Miller SA, Humphries RM. 2014. Comparison of Illumigene, Simplexa, and AmpliVue *Clostridium difficile* molecular assays for diagnosis of *C. difficile* infection. *J. Clin. Microbiol.* 52:960–963. <http://dx.doi.org/10.1128/JCM.02354-13>.
 15. Lalande V, Barrault L, Wadel S, Eckert C, Petit JC, Barbut F. 2011. Evaluation of a loop-mediated isothermal amplification assay for diagnosis of *Clostridium difficile* infections. *J. Clin. Microbiol.* 49:2714–2716. <http://dx.doi.org/10.1128/JCM.01835-10>.
 16. Bamber AI, Fitzsimmons K, Cunliffe JG, Beasor CC, Mackintosh CA, Hobbs G. 2012. Diagnosis of *Clostridium difficile*-associated disease: examination of multiple algorithms using toxin EIA, glutamate dehydrogenase EIA and loop-mediated isothermal amplification. *Br. J. Biomed. Sci.* 69:112–118.
 17. Walkty A, Lagacé-Wiens PRS, Manickam K, Adam H, Pieroni P, Hoban D, Karlowicz JA, Alfa M. 2013. Evaluation of an algorithmic approach in comparison with the Illumigene assay for laboratory diagnosis of *Clostridium difficile* infection. *J. Clin. Microbiol.* 51:1152–1157. <http://dx.doi.org/10.1128/JCM.03203-12>.
 18. Squire MM, Carter GP, Mackin KE, Chakravorty A, Norén T, Elliott B, Lyras D, Riley TV. 2013. Novel molecular type of *Clostridium difficile* in neonatal pigs, western Australia. *Emerg. Infect. Dis.* 19:790–792. <http://dx.doi.org/10.3201/eid1905.121062>.
 19. Geric Stare B, Rupnik M. 2010. *Clostridium difficile* toxinotype XI (A-B-) exhibits unique arrangement of PaLoc and its upstream region. *Anaerobe* 16:393–395. <http://dx.doi.org/10.1016/j.anaerobe.2010.04.001>.
 20. Planche TD, Davies KA, Coen PG, Finney JM, Monahan IM, Morris KA, O'Connor L, Oakley SJ, Pope CF, Wren MW, Shetty NP, Crook DW, Wilcox MH. 2013. Differences in outcome according to *Clostridium difficile* testing method: a prospective multicentre diagnostic validation study of *C. difficile* infection. *Lancet Infect. Dis.* 13:936–945. [http://dx.doi.org/10.1016/S1473-3099\(13\)70200-7](http://dx.doi.org/10.1016/S1473-3099(13)70200-7).