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tcdA As a Diagnostic Target in a Loop-Mediated Amplification Assay for Detecting Toxigenic *Clostridium difficile*

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Background: The *illumi*gene® (Meridian Bioscience, Inc., Cincinnati, OH) and GeneOhm® (BD Diagnostics, La Jolla, CA) *Clostridium difficile* assays target the *tcdA* gene and *tcdB* gene, respectively. We assessed the use of *tcdA* as the molecular target in the *illumi*gene® *C. difficile* loop-mediated amplification assay in detecting a wide variety of *C. difficile* strains including those with *tcdA* deletions. *Methods:* We tested 38 *C. difficile* strains and 108 patient stool specimens using the *illumi*gene® assay. The GeneOhm® real-time polymerase chain reaction (PCR) assay served as the reference method. Discordant results were resolved by repeat testing, anaerobic culture, and a laboratory-developed real-time PCR targeting *tcdA* and *tcdB*. *Results:* Both *illumi*gene® and GeneOhm® assays detected all 37 *C. difficile* toxin B^+ strains representing seven toxinotypes and including four toxin A^-B^+ isolates. No cross-reactivity with 20 other *Clostridium* species or toxin-negative *C. difficile* was observed in either assay. Among patient stool specimens, agreement was 94.4% (102/108). After discordant result resolution, agreement was 96.3% (104/108). Specimens with initially discordant results had target concentrations approaching the limit of detection for the two commercial assays. Discordance appeared unrelated to whether *tcdA* or *tcdB* was the amplification target. *Conclusion:* The *tcdA* 5′ region used by the *illumigene*® assay is a practical target for toxigenic *C. difficile* detection. J. Clin. Lab. Anal. 27:171–176, 2013. © 2013 Wiley Periodicals, Inc.

Key words: *tcdB*; LAMP; *illumi*gene; strains, toxigenic

INTRODUCTION

Toxin-producing *Clostridium difficile* is the most common cause of hospital-associated diarrhea, and community-acquired *C. difficile* infections (CDIs) are rapidly increasing (1). These concerning trends demand rapid diagnosis for treatment and infection control. Many clinical laboratories now use nucleic acid amplification-based methods for detection of toxigenic *C. difficile* in stool samples in lieu of the less-sensitive enzyme immunoassay (2, 3).

Most cases of CDI are caused by strains expressing both toxins A and B (A^+B^+). Toxin A^-B^+ strains, while less frequently encountered, can cause disease and have been implicated in outbreaks of CDI (4–6). Human disease attributable to toxin A^+B^- strains is extremely uncommon (7, 8).

The most common genetic causes for toxin A^-B^+ *C. difficile* strains are a nonsense mutation within *tcdA* (toxinotype VIII) or deletions within conserved sequences at the 3′ end of *tcdA* (toxinotypes X, XVI, and XVI; (8–10)). Integral to the design of molecular assays targeting *tcdA*

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is the fact that its 5′ end is conserved for the majority of known toxin-producing $A^{-}B^{+}$ strains, specifically those causing known outbreaks (4,6,11,12). Sequence analyses of *tcdA* have shown that it is more conserved than *tcdB* (8). Taken together, these observations suggest that targeting the 5′-end of *tcdA* is a promising strategy for detection of clinically relevant *C. difficile* strains.

The *illumi*gene® *C. difficile* assay (Meridian Bioscience, Inc., Cincinnati, OH) is a US Food and Drug Administration (FDA) cleared assay that targets the toxin A gene (*tcdA*), utilizing loop-mediated isothermal amplification (LAMP). Published studies thus far have demonstrated good correlation of the *illumi*gene® assay with other FDA-cleared molecular *C. difficile* assays using clinical specimens (13–18). Although the amplification target of the *illumi*gene® assay is located in the highly conserved 5′-region of *tcdA*, which is present in almost all known toxin $A^{-}B^{+}$ strains, there remains concern that the assay does not amplify the widely preferred amplification target, *tcdB* (16, 19). Toxin B, and not necessarily toxin A, is produced by virtually all pathogenic strains to date. Studies of various toxinotypes of *C. difficile*, including $A^{-}B^{+}$ strains, have also not yet been tested in parallel on commercial amplification assays that target either *tcdA* or *tcdB*.

In this study, we compared the ability of the *illumi*gene® assay and GeneOhm® *C. difficile* assay to recognize various *C. difficile* reference strains representing various toxin classes, and assessed their cross-reactivity with other *Clostridium* species. Using GeneOhm® as a molecular reference method, we evaluated agreement of the *illumi*gene® assay, and utilized culture and a laboratory-developed real-time polymerase chain reaction (PCR) targeting both *tcdA* and *tcdB* as arbitrator methods.

MATERIALS AND METHODS

Clostridium Isolates

Thirty-eight different strains of *C. difficile* provided by Meridian Bioscience originated from various collections where they had previously been characterized (Table 1). Strains were supplied at a density of 4 McFarland standards (~1.2×10^9 CFU/ml). Archived suspensions of 40 *Clostridium* isolates representing 20 different species previously isolated at ARUP Laboratories (Salt Lake City, UT) were subcultured to Columbia blood agar (Hardy Diagnostics, Santa Maria, CA) and incubated anaerobically (Table 2). These consisted of *C. aldenense* ($n = 2$), *C. bifermentans* ($n = 4$), *C. butyricum* ($n = 1$), *C. citroniae* ($n = 1$), *C. clostridioforme* ($n = 2$), *C. glycolicum* ($n = 2$), *C. hathewayi* ($n = 5$), *C. hiranonis* ($n = 2$), *C. innocuum* ($n = 1$), *C. novyi* ($n = 1$), *C. paraputrificum* ($n = 1$), *C. peptidivorans* ($n = 2$), *C. perfringens* ($n = 2$), *C. ramosum* ($n = 3$), *C. septicum* ($n = 2$), *C. sordellii* ($n = 3$), *C. sporogenes* ($n = 2$), *C. symbiosum* ($n = 1$), *C. tertium* ($n = 1$), and *C. xylanolyticum*/*C. aerotolerans* ($n = 2$). Colonies were suspended in phosphate-buffered saline (PBS) to a density of 4 McFarland standards (~1.2×10^9 CFU/ml) prior to testing. All resuspended colony isolates were tested by the *illumi*gene® and GeneOhm® *C. difficile* assays within 24–72 h of each other. Both assays were performed as directed by the package insert except using 50 μl colony suspensions as the starting material instead of stool.

Patient Stool Specimens

This study was approved under the University of Utah Institutional Review Board. Unselected diarrheal stool specimens ($n = 108$) submitted to ARUP Laboratories for *C. difficile* detection were tested with the GeneOhm® assay (BD Diagnostics, La Jolla, CA) as per the manufacturer's package insert. Testing was performed within 48 h of specimen collection and samples were stored at 4°C. Testing with the *illumi*gene® assay was performed within 24–72 h of initial testing and within the specimen stability limits according to the package insert instructions (5 days at 4°C). For future discrepant analysis, specimens were stored at −20°C per kit instructions and in-house validation studies of specimen stability.

Analysis of Discrepant Results

Analysis of discrepant results differed slightly between *Clostridium* isolates and patient stool samples. *Clostridium* isolates that yielded discrepant results between the GeneOhm® and *illumi*gene® assays were repeated on both platforms. They were also subcultured on Brucella blood agar (Hardy Diagnostics) and incubated anaerobically at 37°C for 24–48 h. If more than one colony type was observed, each colony type was isolated for further analysis.

Patient stool specimens with discrepant results between the two commercial assays were thawed to room temperature and tested again with both assays. Extracts from each assay were also subjected to a laboratory-developed real-time PCR (see below). Stool specimens were plated on cycloserine-cefoxitin-fructose agar (CCFA; Hardy Diagnostics) and grown anaerobically at 37°C for 24–72 h. Putative *C. difficile* colonies, as identified by characteristic Gram stain, colony morphology and odor, and fluorescence under Wood's lamp were isolated by subculturing on Brucella blood agar. In one case, the sample did not yield any *C. difficile* or other bacterial colonies. In a further attempt to isolate *C. difficile*, the specimen was subjected to ethanol shock treatment by mixing with 100% ethanol at a ratio of 1:1, vortexing, and incubating at ambient

TABLE 1. Source, Toxinotype and Results With the *illumi*gene® and GeneOhm® for 38 *C. difficile* Strains

C. difficile strain	Source	Toxinotype	Toxin produced	*illumigene*® result	GeneOhm® result
11186	VPI collection	N/A	None	Neg	Neg
10463	VPI collection	0	A^+ B^+	Pos	Pos
2004111	CDC	0	A^+ B^+	Pos	Pos
2004205	CDC	0	A^+ B^+	Pos	Pos
2005070	CDC	0	A^+ B^+	Pos	Pos
2005257	CDC	0	A^+ B^+	Pos	Pos
2008029	CDC	0	A^+ B^+	Pos	Pos
2008162	CDC	0	A^+ B^+	Pos	Pos
2008341	CDC	0	A^+ B^+	Pos	Pos
2008351	CDC	0	A^+ B^+	Pos	Pos
2009066	CDC	0	A^+ B^+	Pos	Pos
2009099	CDC	0	A^+ B^+	Pos	Pos
B1	Hines VA Hosp.	0	A^+ B^+	Pos	Pos
G1	Hines VA Hosp.	0	A^+ B^+	Pos	Pos
J7	Hines VA Hosp.	0	A^+ B^+	Pos	Pos
K12	Hines VA Hosp.	0	A^+ B^+	Pos	Pos
Y1	Hines VA Hosp.	0	A^+ B^+	Pos	Pos
2004052	CDC	III	A^+ B^+	Pos	Pos
2004118	CDC	III	A^+ B^+	Pos	Pos
2007431	CDC	III	A^+ B^+	Pos	Pos
2009052	CDC	III	A^+ B^+	Pos	Pos
BI17	Hines VA Hosp.	III	A^+ B^+	Pos	Pos
BI8	Hines VA Hosp.	III	A^+ B^+	Pos	Pos
2007858	CDC	IX/XXIII	A^+ B^+	Pos	Pos
2005325	CDC	V	A^+ B^+	Pos	Pos
2006240	CDC	V	A^+ B^+	Pos	Pos
2008188	CDC	V	A^+ B^+	Pos	Pos
2009065	CDC	V	A^+ B^+	Pos	Pos
BK6	Hines VA Hosp.	V	A^+ B^+	Pos	Pos
2009018	CDC	V	A^+ B^+	Pos	Pos
43598	ATCC	VIII	A^- B^+	Pos	Pos
2008016	CDC	VIII	A^- B^+	Pos	Pos
CF1	Hines VA Hosp.	VIII	A^- B^+	Pos	Pos
8864	S.P. Borriello	X	A^- B^+	Pos	Pos
2007435	CDC	XII	A^+ B^+	Pos	Pos
2009132	CDC	Unknown	Unknown	Pos	Pos
2009155	CDC	Unknown	Unknown	Pos	Pos
2009277	CDC	Unknown	Unknown	Pos	Pos

Note: VPI, Virginia Polytechnic Institute; CDC, Centers for Disease Control and Prevention; VA, Veterans Affairs; ATCC, American Type Culture Collection.

temperature for 30 min. The sample was then plated on CCFA and resulting colonies were isolated as described above.

All isolated colonies were suspended in PBS to a density of 4 McFarland (~1.2 × 10^9 CFU/ml) prior to molecular testing. Suspensions were tested with both the GeneOhm® and *illumi*gene® assays as described above.

Laboratory-Developed Real-Time PCR Analysis

Two real-time PCR assays targeting *tcdA* and *tcdB* were developed to resolve discrepant results. PCR reactions (25 μl final volume) contained 2.0 μl isolate suspensions or specimen lysates from the commercial assays, 5× QuantiTect Multiplex PCR mastermix (Qiagen, Valencia, CA), 0.5 U AmpErase uracil N-glycosylase (Life Technologies, Carlsbad, CA), *tcdA* or *tcdB* primers and probes (Table 3; Epoch Bioscience, Bothell, WA), 1,000 copies internal control template, and internal control primers and probes (20). Resulting amplicon lengths were 79 bp for *tcdA* and 53 bp for *tcdB*. Pleiades probes containing 5′ MGB and FAM fluorophores and a 3′ BHQ quencher were used for amplicon detection (21). Reactions were carried out on the ABI7900HT instrument (Life Technologies) under the following cycling conditions: 50°C for 2 min, 95°C for 8 min, followed by 50 cycles of 95°C for 15 sec, 58°C for 30 sec, and 76°C for 30 sec, and ending with a dissociation curve analysis (15-sec holds at 95°C, 45°C, and 95°C).

TABLE 2. Primer and Probe Sequences Utilized by the Laboratory-Developed Real-Time PCR for Discrepant Results Analysis

Primer/probe name	Sequence	Concentration (μM)
tcdA–forward	5′-AATAAATCATAAA*TGGT* T*T*ACCTCA*GATAG-3′	0.25
tcdA–reverse	5′-AATAAATCATAAGTTAGCA* TCCGTA*TTAGCAG-3′	1.00
tcdB–forward	5′- AATAAATCATAACCA*GTA* AAATCA*AT*T*GCT-3′	1.00
tcdB–reverse1	5′-AATAAATCATAACCA*GCT AATA*CACT*T*GATGA-3′	0.25
tcdB–reverse2	5′-AATAAATCATAACCA*GCA GATA*CACT*T*GATGA-3′	0.25
tcdA probe	5′-G*AATACTTTGCA CCTGC-3′	0.20
tcdB probe	5′-CTAGAAGGN* GA*A*GCA* A*-3′	0.20

Note: A*, Super A®; G*, Super G®; N*, universal base (proprietary bases are trademarks of Epoch Bioscience).

TABLE 3. Agreement Between the GeneOhm® and *illumi*gene® *C. difficile* Assays With 108 Clinical Specimens

*illumi*gene® *C. difficile* assay	GeneOhm® *C. difficile* assay Positive	Negative	Total
Positive	17	1	18
Negative	5	85	90
Total	22	86	108

RESULTS

Results of *Clostridium* spp. Isolates Tested With *Illumi*gene® and GeneOhm®

Thirty-seven toxin-producing *C. difficile* strains and one nontoxin-producing strain yielded expected results and showed 100% concordance between the GeneOhm® and *illumi*gene® assays (Table 1). The *illumi*gene® assay detected all toxin A^+B^+ strains of toxinotypes 0 ($n = 16$), III ($n = 6$), V ($n = 6$), XII ($n = 1$), and IX/XXIII ($n = 1$). It also amplified all toxin A^-B^+ strains of toxinotypes VIII ($n = 3$) and X ($n = 1$).

Neither the *illumi*gene® nor the GeneOhm® assay amplified 40 non-*C. difficile Clostridium* isolates, including the closely related *C. sordellii* ($n = 3$). One *C. sordellii* isolate initially tested as invalid with the *illumi*gene® assay due to internal control failure, but was negative upon retesting. The sample was negative with the GeneOhm® assay. One *C. symbiosum* sample tested initially positive with the *illumi*gene® and negative with the GeneOhm® assay. The laboratory-developed real-time PCR assays confirmed the presence of *tcdA* and *tcdB*. Subculture of the isolate revealed two colony types, one characteristic for *C. difficile* and one for *C. symbiosum*. By the laboratory-developed real-time PCR assay, the presumptive *C. difficile* isolate was positive for *tcdA* and *tcdB* and the other colony type was negative for *tcdA* and *tcdB*. Repeat testing on the *illumi*gene® and GeneOhm® assays confirmed these results.

Analysis of Clinical Specimens With *Illumi*gene® and GeneOhm®

There was an initial concordance of 94.4% (102/108) between the GeneOhm® and *illumi*gene® assays with the patient samples (Table 3). Using the GeneOhm® assay as the reference method, the sensitivity and specificity of the *illumi*gene® assay was 77.3% (17/22) and 98.8% (85/86), respectively.

Six samples (cases 1–6) with discordant results were retested with both assays (Table 4). Two *illumi*gene®-negative/GeneOhm®-positive specimens (cases 4 and 5) tested positive with both assays upon retesting. One specimen (case 1) was GeneOhm®-negative/*illumi*gene®-positive. The lysate from the *illumi*gene® assay tested positive for *tcdA* and *tcdB* with the laboratory-developed real-time PCR. In contrast, the specimen lysate from the GeneOhm® assay tested negative for *tcdB* and positive for *tcdA*. Upon retesting, the GeneOhm® assay was still negative but the lysate was positive for *tcdA* and *tcdB* by the laboratory-developed real-time PCR. *Clostridium difficile* colonies were isolated from the original stool specimen and were positive on both commercial assays. In two of the remaining three specimens (cases 2 and 3), both GeneOhm®-positive and *illumi*gene®-negative, *C. difficile* colonies were isolated and tested positive with both commercial assays. In the third specimen (case 6), positive GeneOhm® and negative *illumi*gene® results were confirmed during repeat testing. Testing of lysates from both assays with the laboratory-developed real-time PCR confirmed the presence of *tcdB* but not *tcdA*. Culture for *C. difficile* from the original specimen yielded two colonies characteristic for *C. difficile* after ethanol shock treatment. Both colonies tested negative with both commercial assays. In all cases with discordant results, the specimen lysates from both commercial assays were found to be near the limit of detection (LOD; ten copies per reaction) when tested with the laboratory-developed real-time *tcdB* PCR.

DISCUSSION

Most commercially available and laboratory-developed molecular tests or toxigenic *C. difficile* target *tcdB* because toxin B production appears to be ubiquitous in clinically relevant strains (3,22,23). The *illumi*gene® assay is unique among currently US FDA cleared molecular assays for *C. difficile* in that it targets *tcdA*. This assay detected all 37 toxigenic *C. difficile* reference strains used in this study.

TABLE 4. Discrepant Result Analysis

	BD GeneOhm®			*Illumi*gene®			Laboratory-developed PCR		
Case	Initial specimen lysate	Repeat specimen lysate	Isolate	Initial specimen lysate	Repeat specimen lysate	Isolate	*tcdA*	*tcdB*[a]	Resolved result after discordant analysis
1	NEG	NEG	POS	POS	POS	POS	POS	POS[b]	Low positive
2	POS	POS	POS	NEG	NEG	POS	POS	POS	Low positive
3	POS	POS	POS	NEG	NEG	POS	POS	POS	Low positive
4	POS	POS	POS	NEG	POS	POS	POS	POS	Low positive
5	POS	POS	POS	NEG	POS	POS	POS	POS	Low positive
6	POS	POS	NEG[c]	NEG	NEG	NEG[c]	NEG	POS	Presumptive low positive

[a]All specimens contained less than ten copies per reaction as determined by the laboratory-developed real-time PCR assay.
[b]The *tcdB* PCR was initially negative with the GeneOhm® lysate, but positive with specimen lysate from repeat testing.
[c]Two *C. difficile* colonies could be isolated and were tested.

Four toxin A^-B^+ isolates were available for testing covering the two most clinically relevant A^-B^+ toxinotypes (VIII and X) that have caused outbreaks (4, 6, 11, 12). It is important to note that emerging toxinotypes may have deletions in *tcdA* that could affect clinical assays. In a recent study, newly described A^-B^+ toxinotypes XXX and XXXI were not detected by the *illumi*gene® assay due to deletions in *tcdA* (24). They represented 0.6% and 0.1% of *C. difficile* isolates in the study leading to their discovery, suggesting they are uncommon strains at this time (19). Overall, the *illumi*gene® assay was able to detect the most clinically relevant strains of *C. difficile*.

Analysis of 108 stool specimens with both commercial assays showed good agreement (94.4%). Discrepant results were most likely caused by specimens containing a low number of toxigenic *C. difficile* in combination with sampling error and/or differences in the analytical sensitivities of the assays. Specimen lysates generating a GeneOhm®-positive/*illumi*gene®-negative or GeneOhm®-negative/*illumi*gene®-positive result were tested with the laboratory-developed real-time PCR and found to contain approximately ten copies of *tcdB* per reaction. This observation suggests the presence of toxigenic *C. difficile* at a concentration approaching the LOD of the GeneOhm® assay (4 CFU/reaction or ten copies per reaction) and the *illumi*gene® assay (4–64 CFU/reaction). While stool specimens were all tested within the stated stability limits of the commercial assays, they were tested by the *illumi*gene® assay 24–72 h after being tested by the GeneOhm® assay. This delay may have contributed to the occurrence of GeneOhm®-positive/*illumi*gene®-negative results in five of the six discordant cases. In one specimen that was repeatedly GeneOhm®-positive and *illumi*gene®-negative (Table 4, case 6), the laboratory-developed real-time PCR of the specimen lysate was positive for *tcdB* and negative for *tcdA* while the cultured isolate (two colonies) was negative for both *tcdA* and *tcdB*. These inconclusive results may be explained by the presence of both toxigenic and nontoxigenic *C. difficile* strains in low numbers in the stool specimen, the difference in the LOD of our laboratory-developed PCR assay for *tcdA* compared to *tcdB*, or (less likely) a *C. difficile* strain with a deletion or mutation in *tcdA* that precluded detection by the laboratory-developed real-time PCR *tcdA* and the *illumi*gene® assays.

The clinical significance of low bacterial loads for toxigenic *C. difficile* in diarrheal stool samples remains unclear. Since toxigenic *C. difficile* is known to colonize patients without causing symptoms, and CDI is attributable to multiple factors, such as host immune response and gut microbiota, detection of the *C. difficile* toxin genes does not equate infection (25, 26). Unfortunately, we were unable to determine possible alternative causes for the diarrheal disease in these patients since specimens were referred for analysis from a number of hospitals throughout the United States and clinical histories were not available.

Conclusion

The ability to rapidly and accurately diagnose CDI is important for prompt treatment and implementation of infection control measures. Targeting *tcdA* at the 5′ conserved region using a LAMP-based assay allowed accurate detection of both toxin A^+B^+ and most clinically relevant toxin A^-B^+ *C. difficile* strains encompassing more than seven toxinotypes. Both the GeneOhm® and the *illumi*gene® *C. difficile* assays accurately identified toxin-producing *C. difficile* strains and were highly specific when tested against a toxin-negative strain and 20 other *Clostridium* species. The *illumi*gene® *C. difficile* assay was easy to use and appears to offer similar performance characteristics as other commercially available molecular assays such as the BD GeneOhm®.

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