



Multiplex Real-Time PCR Method for Simultaneous Identification and Toxigenic Type Characterization of *Clostridium difficile* From Stool Samples

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Background: The aim of this study was to develop and validate a multiplex real-time PCR assay for simultaneous identification and toxigenic type characterization of *Clostridium difficile*.

Methods: The multiplex real-time PCR assay targeted and simultaneously detected triose phosphate isomerase (*tpi*) and binary toxin (*cdtA*) genes, and toxin A (*tcdA*) and B (*tcdB*) genes in the first and second tubes, respectively. The results of multiplex real-time PCR were compared to those of the BD GeneOhm Cdiff assay, targeting the *tcdB* gene alone. The toxigenic culture was used as the reference, where toxin genes were detected by multiplex real-time PCR.

Results: A total of 351 stool samples from consecutive patients were included in the study. Fifty-five stool samples (15.6%) were determined to be positive for the presence of *C. difficile* by using multiplex real-time PCR. Of these, 48 (87.2%) were toxigenic (46 *tcdA* and *tcdB*-positive, two positive for only *tcdB*) and 11 (22.9%) were *cdtA*-positive. The sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) of the multiplex real-time PCR compared with the toxigenic culture were 95.6%, 98.6%, 91.6%, and 99.3%, respectively. The analytical sensitivity of the multiplex real-time PCR assay was determined to be 103 colony-forming unit (CFU)/g spiked stool sample and 0.0625 pg genomic DNA from culture. Analytical specificity determined by using 15 enteric and non-clostridial reference strains was 100%.

Conclusions: The multiplex real-time PCR assay accurately detected *C. difficile* isolates from diarrheal stool samples and characterized its toxin genes in a single PCR run.

Key Words: Multiplex real-time PCR, *Clostridium difficile*, Toxin variant strains, Binary toxin

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INTRODUCTION

Clostridium difficile is a gram-positive, spore-forming, obligate anaerobe. This bacterium is the causative organism for *C. difficile* infection (CDI), which presents symptoms ranging from mild diarrhea to toxic megacolon, ileus, bowel perforation, pseudomembranous colitis, and even death [1]. The major determinants of virulence in *C. difficile* are toxin A and toxin B, two members of

the large clostridial cytotoxin family. Toxins A and B are encoded by the genes *tcdA* and *tcdB*, respectively, which are located within a 19.6-kb region of the chromosome known as the pathogenicity locus, or PaLoc [2]. Although most pathogenic strains of *C. difficile* produce both toxins, toxin A-negative, toxin B-positive variant strains have also been reported worldwide [3, 4]. Some studies have also reported the presence of another toxin, a binary toxin produced by certain strains of *C. difficile*. This toxin is en-

coded by the genes *cdtA* (enzymatic component) and *cdtB* (binding component), which are located outside the PaLoc, at the CDT locus in the genome [5].

Several laboratory methods have been used for diagnosis of CDI, including enzyme immunoassays (EIAs) for toxins A and/or B, EIAs for glutamate dehydrogenase (GDH), cell cytotoxin neutralization assay (CCNA), toxigenic culture, immunochromogenic assay, and real-time PCR methods. Direct stool culture and CCNA are currently the reference standard methods for the diagnosis of CDI. However, both methods are laborious, time consuming, and must be performed by qualified, trained personnel. EIAs are used for CDI diagnosis in over 90% of the laboratories in the United States because of their relative speed, ease of operation, and low-cost, compared with the reference standard assay. However, EIAs are not sensitive enough [6]. Recently, commercial and in-house-developed real-time PCR methods have been developed for the detection of *C. difficile* toxin genes from stool samples. These methods detect the genes with high sensitivity, specificity, and low turnaround time. Commercially available products for *C. difficile* diagnosis include the BD GeneOhm Cdiff (BD Diagnostics; San Diego, CA, USA), Prodesse ProGastro Cd (Gen-Probe Inc.; San Diego, CA, USA), Xpert *C. difficile* (Cepheid; Sunnyvale, CA, USA), and illumigene *C. difficile* (Meridian Biosciences; Cincinnati, OH, USA) real-time PCR tests. All the tests target the *tcdB* gene, except for the illumigene *C. difficile* test, which targets the *tcdA* gene. The Xpert *C. difficile*/Epi (Cepheid) is additionally capable of detecting the binary toxin. Compared with the CCNA and/or toxigenic culture, the PCR assays were reported with sensitivities and specificities ranging from 77% to 100% and 93% to 100%, respectively. However, none of the commercially available real-time PCR tests are capable of simultaneously targeting genes for the identification of the organism, toxin A, toxin B, and the binary toxin [6-9].

The purpose of this study was to develop and evaluate a multiplex real-time PCR assay for the simultaneous identification and toxigenic type characterization of *C. difficile* isolates. In addition, the assay characteristics were compared with those of the reference method (toxigenic culture) and a commercially available PCR assay (BD GeneOhm Cdiff assay, targeting *tcdB* gene).

METHODS

1. Patients and samples

The prospective study was conducted at a university-affiliated,

800+ bed hospital in the Texas Medical Center, Houston, TX. Three hundred seventy-five consecutive liquid or soft stool specimens submitted to the Clinical Microbiology Department for *C. difficile* testing between February 20 and March 9, 2012 were considered for this study. Among these, only the first sample obtained from each unique patient was used. A total of 351 consecutive stool samples were tested by toxigenic culture, multiplex real-time PCR, and the standard BD PCR. The BD GeneOhm Cdiff assay (the standard diagnostic test used by the clinical microbiology laboratory) and the multiplex real-time PCR assay were performed within 24 hr after collection. The specimens were also cultured for *C. difficile* within 24 hr after collection of the stool samples. An aliquot of each sample was also frozen at -20°C for any required re-culturing. This study was approved by the St. Luke's Episcopal Hospital Institutional Review Board.

2. DNA extraction and BD GeneOhm Cdiff PCR assay

The BD GeneOhm Cdiff assay was performed according to the manufacturer's instructions. Briefly, the diarrheal stool samples were vortexed at high speed. The stool specimens were transferred into the sample buffer tube containing Tris-EDTA sample preparation buffer by using sterile dacron swabs, which were broken into the tubes. The sample buffer tube containing the broken swab was tightly closed and vortexed at high speed for 1 min. Forty microliters of un-inoculated sample buffer was transferred to the lysis tube with glass beads, to which 10 µL of sample buffer containing the stool sample suspension was added. The lysis tube was vortexed for 5 min at high speed in order to ensure settling of the contents at the bottom of the tube. The DNA samples were inactivated at 95°C in a dry block for 5 min, and immediately placed on ice. These samples were used for the BD GeneOhm Cdiff PCR and the multiplex real-time PCR assays. Three microliters of each DNA sample and positive and negative controls were added to the respective, labeled SmartCycler tube, containing 25 µL of the reconstituted master mix. The reaction tubes were centrifuged for 10 sec, run by using the SmartCycler I-CORE module (Cepheid), and analyzed with the Cepheid SmartCycler software, by using the standard BD GeneOhm Cdiff assay amplification protocol [10].

3. Design of primers and probes for the multiplex real-time PCR assay

The oligo analysis and design program (Oligoware 3.0 GMMA; Ankara, Turkey) was used to design of the *tpi* (species-specific internal fragment of the triose phosphate housekeeping gene), *tcdA*, *tcdB*, and *cdtA* primers and probes (Table 1). The se-

Table 1. The multiplex real-time PCR primers and TaqMan probes for detection of *Clostridium difficile*, its specific toxins A and B, and binary toxin A genes

Main target	Target genes	Sequence	GenBank accession number
<i>C. difficile</i>	<i>tpi</i>	F: 5'-gaagctactaagggtacaaa-3'	FN668944
		R: 5'-ggctattcctacttctaagc-3'	FN668941
		Probe: 5'-VIC ataagaggtgaaacttctctgtaaagtctcc TAMRA-3'	FN668375
			FN665654
			FN665653
Toxin A	<i>tcdA</i>	F: 5'-tgataacgtatagcttgacc-3'	DQ117264
		R: 5'-atggtttacctcagatagg-3'	DQ117255
		Probe: 5'-FAM tgaatactttgcacctgctaatacggatg TAMRA-3'	DQ117249
			DQ117248
			DQ117247
Toxin B	<i>tcdB</i>	F: 5'-gaaggattacctgtaattgc-3'	HM062511
		R: 5'-ctgccattatacctatcttagc-3'	HM062510
		Probe: 5'-VIC ctctttgattgctgcacctaaacttacacc TAMRA-3'	HM062509
			HM062508
			HM062507
Binary toxin A	<i>cdtA</i>	F: 5'-tatattaagcagaagcatctgt-3'	DQ102377
		R: 5'-ctggaccatttgatattaataatt-3'	DQ102378
		Probe: 5'-FAM tcctccacgcataatacattacatcagc TAMRA-3'	DQ102375
			DQ102376
			AJ238325

Abbreviations: *tpi*, *Clostridium difficile* specific triose phosphate isomerase gene; *tcdA*, toxin A gene; *tcdB*, toxin B gene; *cdtA*, binary toxin gene.

quences were evaluated by using the Basic Local Alignment Search Tool (BLAST) in order to assess the specificity of the primers and probes towards the identification of target sequences. The primers and probes were synthesized by Applied Biosystems (Foster City, CA, USA).

4. Multiplex real-time PCR for *C. difficile* identification and toxin detection

The multiplex real-time PCR assay was designed for the targeting and detection of the *tpi* and *cdtA*, and *tcdA* and *tcdB* genes in the first and second tubes, respectively, in a single PCR run. One microliter of the DNA sample was added to each of the two tubes containing 24 μ L of the reaction mixture (0.8 μ M of each primer, 0.4 μ M of each fluorophore probe, 6 mM MgCl₂, 200 μ M dNTPs, 1 IU Super-Hot Taq polymerase, and 1 \times PCR buffer; Applied Biosystems). The assay was run on the ViiA 7 real-time PCR System (Applied Biosystems). The cycle conditions set for the use of the hydrolysis probe were: denaturation of the pre-amplified templates at 95°C for 15 min, followed by 45 cycles of denaturation at 95°C for 15 sec, and annealing and extension at

60°C for 60 sec.

5. Determination of sensitivity and specificity of the multiplex real-time PCR assay

The bacterial reference strains used in the analytical sensitivity and specificity tests are listed in Table 2. The analytical sensitivity of the assay was determined by using spiked stool specimens. A bacterial suspension, approximately 10² to 10⁹ colony forming units (CFU)/mL, was obtained from an overnight culture of the *C. difficile* R20291 (BI/NAP1/027) toxigenic strain. Suspension aliquots (0.1 mL) were transferred into 0.9 mL *C. difficile*-negative stool samples. DNA was extracted from bacteria isolated from the stool samples at concentrations ranging from 10¹ to 10⁸ CFU/g [10]. This extracted DNA was used to determine the analytical sensitivity of the multiplex real-time PCR assay. The analytical sensitivity of the multiplex real-time PCR was determined by using two-fold serial dilutions of the extracted genomic *C. difficile* R20291 (BI/NAP1/027) toxigenic strain (DNA concentrations ranging from 0.015625 pg to 4 pg). The assay was performed on the ViiA 7 real-time PCR System (Applied Biosystems)

Table 2. Bacterial strains used for preliminary validation of the real-time-*tpi-tcdA-tcdB-cdtA* PCR assay

Organisms	Multiplex PCR results*			
	<i>tpi</i>	<i>tcdA</i>	<i>tcdB</i>	<i>cdtA</i>
<i>Clostridium difficile</i> R20291 (BI/NAP1/027), <i>C. difficile</i> CD196 (BI/NAP1/027), <i>C. difficile</i> M120 (BK/NAP7,8,9/078), <i>C. difficile</i> CD305 (023)	+	+	+	+
<i>C. difficile</i> ATCC 9689, <i>C. difficile</i> BI-9 (J/NAP2/001), <i>C. difficile</i> Liv024 (J/NAP2/001), <i>C. difficile</i> Liv022 (DH/NAP11/106), <i>C. difficile</i> TL178 (G/NAP6/002), <i>C. difficile</i> TL176 (Y/NAP4/014), <i>C. difficile</i> TL174 (015), <i>C. difficile</i> 630 (R/012)	+	+	+	-
<i>C. difficile</i> CF5 (CF/NAP9/017), <i>C. difficile</i> M68 (CF/NAP9/027)	+	-	+	-
<i>Klebsiella pneumoniae</i> ATCC 13883, <i>Pseudomonas aeruginosa</i> ATCC 27853, <i>Escherichia coli</i> ATCC 25922, <i>Staphylococcus aureus</i> ATCC 29213, <i>Campylobacter jejuni</i> ATCC 33291, <i>Enterococcus casseliflavus</i> ATCC 700327, <i>Streptococcus pyogenes</i> ATCC 19615, <i>Enterobacter aerogenes</i> ATCC 13048, <i>Acinetobacter anitratus</i> ATCC 19606, <i>Salmonella typhi</i> ATCC 14028, <i>Shigella flexneri</i> ATCC 12022, <i>Enterococcus faecalis</i> ATCC 29212, <i>Acinetobacter baumannii</i> BAA 747, <i>Streptococcus pneumoniae</i> ATCC 46619	-	-	-	-

**tpi*, *Clostridium difficile* specific triose phosphate isomerase gene; *tcdA*, toxin A gene; *tcdB*, toxin B gene; *cdtA*, binary toxin gene.
 +, positive result; -, negative result.

by using the same reactants and run conditions that were described above. The cycle of quantification (Cq) value for each dilution was recorded. The lowest concentration of DNA obtained from the spiked stool and pure *C. difficile* R20291 (BI/NAP1/027) samples was identified as the analytical sensitivity of the assay. The PCR efficiency for each assay was determined from the slopes of standard curves ($E = 2^{-1/\text{slope}}$). A panel of 14 non-clostridial isolates was used to determine the analytical specificity (Table 2). Pearson's chi-square and Fisher's exact tests were used to compare sensitivity, specificity, positive and negative predictive values of two tests (BD GeneOhm Cdiff assay vs. the multiplex real-time PCR assay).

6. Toxigenic culture and detection of toxin genes

The stool samples were directly cultured onto cycloserine-cefoxitin-fructose agar (CCFA) plates (Anaerobe Systems; Morgan Hill, CA, USA). The plates were incubated at 37°C for up to 5 days in anaerobic conditions and examined daily for growth. *C. difficile* was identified on the basis of typical odor and colony morphology, and confirmed by a rapid latex agglutination test (*C. difficile*, Oxoid Ltd.; Hampshire, UK) [4]. Following identification of the isolates, a 0.5 McFarland suspension of the *C. difficile* isolate was prepared, and 1 µL of this suspension was used for the detection of *tpi*, *cdtA*, *tcdA*, and *tcdB* genes by multiplex real-time PCR. The assay was performed as described in the previous section. A toxigenic strain was defined as a *C. difficile* isolate expressing either *tcdA* or *tcdB*, or both genes.

RESULTS

The multiplex real-time PCR assay identified 48 out of the total 351 (13.6%) samples as being positive for toxigenic *C. difficile*

(46 positive for both *tcdA* and *tcdB*, two positive for only *tcdB*), whereas the toxigenic culture identified 46 toxigenic *C. difficile*-positive (13.1%) samples (44 positive for *tcdA* and *tcdB*, two for *tcdB* only). The BD GeneOhm PCR assay, on the other hand, identified 52 out of 351 *C. difficile*-positive (14.8%) samples.

Twelve samples (3.4%) were defined as non-toxigenic *C. difficile* (only positive for *tpi* expression) by toxigenic culture, while the multiplex real-time PCR assay classified 11 (3.1%) samples as non-toxigenic *C. difficile*.

A total of 11 *C. difficile* isolates were positive for *cdtA* gene expression, as well as expression of *tcdA* and *tcdB* genes, by the multiplex real-time PCR assay and toxigenic culture.

The analytical sensitivity of the multiplex assay was found to be the same for all targeted genes, at 10³ CFU/g stool (from stool samples) and 0.0625 pg genomic DNA (from culture genomic extraction) (Fig. 1). The multiplex real-time PCR assay yielded negative results with the 14 non-clostridial isolates, demonstrating the absence of cross reactivity (Table 2). The sensitivity, specificity, negative predictive value, and positive predictive value of the multiplex real-time PCR and the BD GeneOhm PCR compared with the toxigenic culture are listed in Table 3. The reaction efficiencies of real-time *tpi*, *cdtA*, *tcdA*, and *tcdB* PCR were found to be 97%, 90%, 105%, and 104%, respectively, with correlation coefficients of over 0.99.

DISCUSSION

The gold-standard methods for diagnosis of CDI include the toxigenic culture and CCNA methods [11]. Both assays are labor-intensive and time-consuming. EIAs that detect toxins A and B are used worldwide, but these assays lack sensitivity. Commercially available real-time PCR assays, with improved sensitivity and

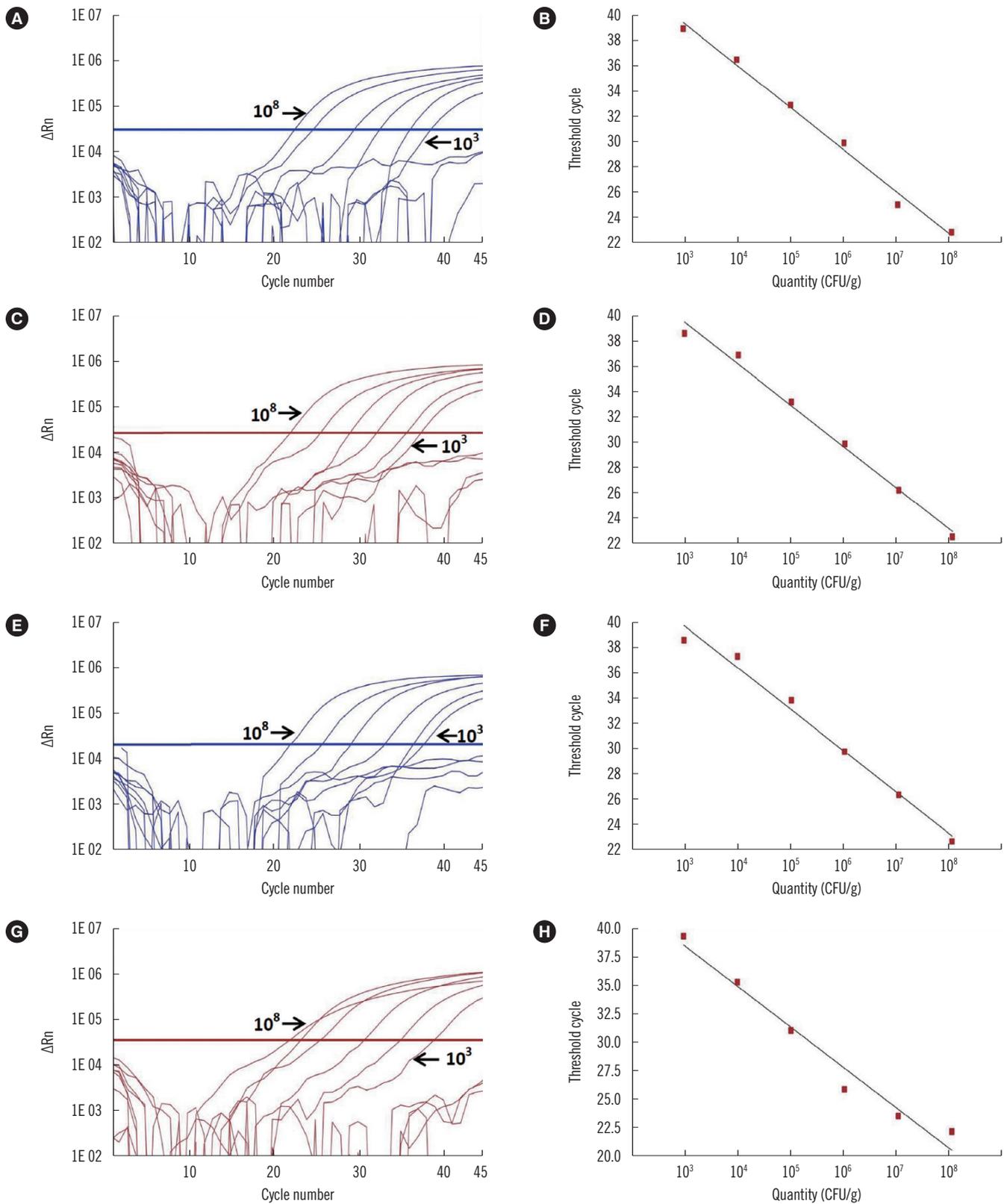


Fig. 1. Amplification curves and dilution end-point standard curves of log₁₀ genome equivalents versus threshold cycle number. The analytical sensitivity of this assay for *tpi* (A-B), *tcdA* (C-D), *tcdB* (E-F), and *cdtA* (G-H) was approximately 10^3 CFU/g. Abbreviations: CFU, colony forming unit; ΔRn , normalized reporter.

Table 3. The BD GeneOhm Cdiff assay and the multiplex real-time PCR assay results compared to toxigenic culture as the reference method

Assay	Toxigenic culture		Assay performance (95% confidence interval)			
	Negative	Positive	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
BD GeneOhm Cdiff						
Negative	298	1	97.8 (87.0-99.8)	97.7 (95.1-98.9)	86.5 (73.5-93.9)	99.6 (97.8-99.9)
Positive	7	45				
Total	305	46				
Real-time PCR						
Negative	301	2	95.6 (83.9-99.2)	98.6 (96.4-99.5)	91.6 (79.1-97.2)	99.3 (97.3-99.8)
Positive	4	44				
Total	305	46				
<i>P</i> value*			0.361	> 0.999	> 0.999	0.413

**P* value for the comparison of sensitivity, specificity, PPV, and NPV of BD GeneOhm Cdiff and Real-time PCR assays.
 Abbreviations: PPV, positive predictive value; NPV, negative predictive value.

faster diagnosis time, have been recently developed. These assays generally target either toxin A or B, and are therefore unable to detect variant strains that produce toxin B only, with or without the binary toxin [12]. In this study, a multiplex real-time PCR assay was developed and validated for the simultaneous detection of genes coding for toxin A, toxin B, binary toxin, and a house-keeping gene for the *C. difficile* organism, from stool samples. The primary advantage of this assay was the simultaneous detection and identification of the *tpi*, *cdtA*, *tcdA*, and *tcdB* genes within two reaction tubes in one PCR run. Performance of the assay was comparable to that of a reference method (toxigenic culture) and a commercially available real-time PCR diagnostic test.

Using the toxigenic culture as reference method, the performance characteristics of the multiplex real-time PCR assay were observed to agree with those seen in previous studies using real-time PCR assays. In this study, four samples determined to be negative in the toxigenic culture were observed to be positive with the real-time PCR assay. Potential reasons for these false-positive PCR results could be a low number of microorganisms in a very heterogeneous stool sample or growth inhibition due to concomitant anti-*C. difficile* treatment. Two samples determined to be negative with the multiplex real-time PCR assay were positive in the toxigenic culture. These samples were repeat-analyzed by both methods, with no change in results. This suggests that some stool samples contained a low bacterial concentration, below the analytical sensitivity of the multiplex real-time PCR. Another reason could be that the modification of the primer and/or hydrolysis probe-binding site sequence within the toxin genes could result in a negative PCR result. The analytical sensitivity of the PCR assay was found to be 10³ CFU/g stool and 0.0625 pg

genomic DNA, results that were 1,000-fold more sensitive than the conventional PCR assay described by Guilbault *et al.* [13], and comparable to other published real-time PCR assays targeting toxin genes of *C. difficile* in stool samples [14-17]. These results suggested that the concentration of *C. difficile* in patient stool samples may be too low. Future research should assess *C. difficile* isolated from patients with CDI at the time of diagnosis, in order to determine the limit of detection required for these emerging assays.

Variant pathogenic *C. difficile* strains containing toxin B but not toxin A have been described, although the clinical characteristics of these strains have not been well studied. This could be due to the lack of identification of these variant strains using assays that detect toxins A or B [18, 19]. The incidence of variant strains ranges from 3% to 92% worldwide [20-26] and from 0.2% to 1.3% in the United States [27, 28]. In this study, two (4.1%) out of 48 toxigenic isolates were discovered to belong to the *tcdA*/*tcdB*+ variant strain by multiplex real-time PCR. Likewise, the binary toxin is associated with the epidemic BI/NAP1/O27 strain, and has recently been shown to be associated with increased mortality [29-31]. The incidence of binary toxin in clinical *C. difficile* isolates varies from 1.6% to 34.6% [5, 32-37]. In this study, the binary toxin gene (*cdtA*) was found in 11 (22.9%) of the 48 toxigenic isolates. This assay provides investigators and clinicians with the ability to simultaneously evaluate these variant strains of *C. difficile*.

This study has certain limitations. The assay was not designed to detect deletions in the *tcdC* gene, a known marker of organisms displaying hypervirulence. Likewise, other known virulence genes in the PaLoc (*tcdE* and *tcdR*) were not targeted. In the fu-

ture, we aim to target these genes in a confirmatory assay that could be performed on all isolates, after identification using the currently described assay. In addition, we did not confirm the binary toxin results with those of any other published assay.

In conclusion, we developed and validated a multiplex real-time PCR stool sample assay for the simultaneous identification and toxigenic type characterization of *C. difficile* isolates. Assay performance characteristics were similar to those for toxigenic culture and a commercially available real-time PCR assay. This assay provides investigators and clinicians with the ability to evaluate variant strains of *C. difficile* simultaneously, including toxin B+A- strains with or without the binary toxin.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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REFERENCES

1. Shah D, Dang MD, Hasbun R, Koo HL, Jiang ZD, DuPont HL, et al. *Clostridium difficile* infection: update on emerging antibiotic treatment options and antibiotic resistance. *Expert Rev Anti Infect Ther* 2010; 8:555-64.
2. O'Connor JR, Johnson S, Gerding DN. *Clostridium difficile* infection caused by the epidemic BI/NAP1/027 strain. *Gastroenterology* 2009; 136:1913-24.
3. Carter GP, Rood JI, Lyras D. The role of toxin A and toxin B in the virulence of *Clostridium difficile*. *Trends Microbiol* 2012;20:21-9.
4. Drudy D, Harnedy N, Fanning S, O'Mahony R, Kyne L. Isolation and characterisation of toxin A-negative, toxin B-positive *Clostridium difficile* in Dublin, Ireland. *Clin Microbiol Infect* 2007;13:298-304.
5. Rupnik M, Grabnar M, Geric B. Binary toxin producing *Clostridium difficile* strains. *Anaerobe* 2003;9:289-94.
6. Tenover FC, Baron EJ, Peterson LR, Persing DH. Laboratory diagnosis of *Clostridium difficile* infection can molecular amplification methods move us out of uncertainty? *J Mol Diagn* 2011;13:573-82.
7. Boyanton BL Jr, Sural P, Loomis CR, Pesta C, Gonzalez-Krellwitz L, Robinson-Dunn B, et al. Loop-mediated isothermal amplification compared to real-time PCR and enzyme immunoassay for toxigenic *Clostridium difficile* detection. *J Clin Microbiol* 2012;50:640-5.
8. Hoegh AM, Nielsen JB, Lester A, Friis-Moller A, Schønning K. A multiplex, internally controlled real-time PCR assay for detection of toxigenic *Clostridium difficile* and identification of hypervirulent strain 027/ST-1. *Eur J Clin Microbiol Infect Dis* 2012;31:1073-9.
9. Lalande V, Barrault L, Wadel S, Eckert C, Petit JC, Barbut F. Evaluation of a loop-mediated isothermal amplification assay for diagnosis of *Clostridium difficile* infections. *J Clin Microbiol* 2011;49:2714-6.
10. Stamper PD, Alcabasa R, Aird D, Babiker W, Wehrlin J, Ikpeama I, et al. Comparison of a commercial real-time PCR assay for *tcdB* detection to a cell culture cytotoxicity assay and toxigenic culture for direct detection of toxin-producing *Clostridium difficile* in clinical samples. *J Clin Microbiol* 2009;47:373-8.
11. Cohen SH, Gerding DN, Johnson S, Kelly CP, Loo VG, McDonald LC, et al. 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* 2010;31:431-55.
12. Norén T, Alriksson I, Andersson J, Akerlund T, Unemo M. Rapid and sensitive loop-mediated isothermal amplification test for *Clostridium difficile* detection challenges cytotoxin B cell test and culture as gold standard. *J Clin Microbiol* 2011;49:710-1.
13. Guibault C, Labbé AC, Poirier L, Busque L, Bêliveau C, Laverdière M. Development and evaluation of a PCR method for detection of the *Clostridium difficile* toxin B gene in stool specimens. *J Clin Microbiol* 2002; 40:2288-90.
14. Barbut F, Monot M, Rousseau A, Cavelot S, Simon T, Burghoffer B, et al. Rapid diagnosis of *Clostridium difficile* infection by multiplex real-time PCR. *Eur J Clin Microbiol Infect Dis* 2011;30:1279-85.
15. Bélanger SD, Boissinot M, Clairoux N, Picard FJ, Bergeron MG. Rapid detection of *Clostridium difficile* in feces by real-time PCR. *J Clin Microbiol* 2003;41:730-4.
16. Kim H, Jeong SH, Kim M, Lee Y, Lee K. Detection of *Clostridium difficile* toxin A/B genes by multiplex real-time PCR for the diagnosis of *C. difficile* infection. *J Med Microbiol* 2012;61:274-7.
17. van den Berg RJ, Kuijper EJ, van Coppenraet LE, Claas EC. Rapid diagnosis of toxigenic *Clostridium difficile* in faecal samples with internally controlled real-time PCR. *Clin Microbiol Infect* 2006;12:184-6.
18. Alfa MJ, Kabani A, Lyerly D, Moncrief S, Neville LM, Al-Barrak A, et al. Characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile* responsible for a nosocomial outbreak of *Clostridium difficile*-associated diarrhea. *J Clin Microbiol* 2000;38:2706-14.
19. Kuijper EJ, de Weerd J, Kato H, Kato N, van Dam AP, van der Vorm ER, et al. Nosocomial outbreak of *Clostridium difficile*-associated diarrhoea due to a clindamycin-resistant enterotoxin A-negative strain. *Eur J Clin Microbiol Infect Dis* 2001;20:528-34.
20. Goorhuis A, Legaria MC, van den Berg RJ, Harmanus C, Klaassen CH, Brazier JS, et al. Application of multiple-locus variable-number tandem-repeat analysis to determine clonal spread of toxin A-negative *Clostridium difficile* in a general hospital in Buenos Aires, Argentina. *Clin Microbiol Infect* 2009;15:1080-6.
21. Kim H, Jeong SH, Roh KH, Hong SG, Kim JW, Shin MG, et al. Investigation of toxin gene diversity, molecular epidemiology, and antimicrobial resistance of *Clostridium difficile* isolated from 12 hospitals in South Korea. *Korean J Lab Med* 2010;30:491-7.
22. Komatsu M, Kato H, Aihara M, Shimakawa K, Iwasaki M, Nagasaka Y, et al. High frequency of antibiotic-associated diarrhea due to toxin A-negative, toxin B-positive *Clostridium difficile* in a hospital in Japan and risk factors for infection. *Eur J Clin Microbiol Infect Dis* 2003;22:525-9.
23. Martin H, Willey B, Low DE, Staempfli HR, McGeer A, Boerlin P, et al. Characterization of *Clostridium difficile* strains isolated from patients in Ontario, Canada, from 2004 to 2006. *J Clin Microbiol* 2008;46:2999-3004.

24. Oka K, Osaki T, Hanawa T, Kurata S, Okazaki M, Manzoku T, et al. Molecular and microbiological characterization of *Clostridium difficile* isolates from single, relapse, and reinfection cases. *J Clin Microbiol* 2012;50:915-21.
25. Pituch H, Rupnik M, Obuch-Woszczatyński P, Grubescic A, Meisel-Mikolajczyk F, Luczak M. Detection of binary-toxin genes (*cdtA* and *cdtB*) among *Clostridium difficile* strains isolated from patients with *C. difficile*-associated diarrhoea (CDAD) in Poland. *J Med Microbiol* 2005;54:143-7.
26. Shin BM, Kuak EY, Yoo SJ, Shin WC, Yoo HM. Emerging toxin A-B+ variant strain of *Clostridium difficile* responsible for pseudomembranous colitis at a tertiary care hospital in Korea. *Diagn Microbiol Infect Dis* 2008;60:333-7.
27. Geric B, Rupnik M, Gerding DN, Grabnar M, Johnson S. Distribution of *Clostridium difficile* variant toxinotypes and strains with binary toxin genes among clinical isolates in an American hospital. *J Med Microbiol* 2004;53:887-94.
28. Lyerly DM, Neville LM, Evans DT, Fill J, Allen S, Greene W, et al. Multi-center evaluation of the *Clostridium difficile* TOX A/B TEST. *J Clin Microbiol* 1998;36:184-90.
29. Bacci S, Mølbak K, Kjeldsen MK, Olsen KE. Binary toxin and death after *Clostridium difficile* infection. *Emerg Infect Dis* 2011;17:976-82.
30. Cartman ST, Heap JT, Kuehne SA, Cockayne A, Minton NP. The emergence of 'hypervirulence' in *Clostridium difficile*. *Int J Med Microbiol* 2010;300:387-95.
31. Persson S, Torpdahl M, Olsen KE. New multiplex PCR method for the detection of *Clostridium difficile* toxin A (*tcdA*) and toxin B (*tcdB*) and the binary toxin (*cdtA/cdtB*) genes applied to a Danish strain collection. *Clin Microbiol Infect* 2008;14:1057-64.
32. Geric B, Rupnik M, Gerding DN, Grabnar M, Johnson S. Distribution of *Clostridium difficile* variant toxinotypes and strains with binary toxin genes among clinical isolates in an American hospital. *J Med Microbiol* 2004;53:887-94.
33. Gonçalves C, Decré D, Barbut F, Burghoffer B, Petit JC. Prevalence and characterization of a binary toxin (actin-specific ADP-ribosyltransferase) from *Clostridium difficile*. *J Clin Microbiol* 2004;42:1933-9.
34. Kim SJ, Kim H, Seo Y, Yong D, Jeong SH, Chong Y, et al. Molecular characterization of toxin A-negative, toxin B-positive variant strains of *Clostridium difficile* isolated in Korea. *Diagn Microbiol Infect Dis* 2010; 67:198-201.
35. Merrigan MM, Sambol SP, Johnson S, Gerding DN. New approach to the management of *Clostridium difficile* infection: colonisation with non-toxicogenic *C. difficile* during daily ampicillin or ceftriaxone administration. *Int J Antimicrob Agents* 2009;33:S46-50.
36. Rupnik M, Kato N, Grabnar M, Kato H. New types of toxin A-negative, toxin B-positive strains among *Clostridium difficile* isolates from Asia. *J Clin Microbiol* 2003;41:1118-25.
37. Samie A, Obi CL, Fransasiak J, Archbald-Pannone L, Bessong PO, Alcantara-Warren C, et al. PCR detection of *Clostridium difficile* triose phosphate isomerase (*tpi*), toxin A (*tcdA*), toxin B (*tcdB*), binary toxin (*cdtA*, *cdtB*), and *tcdC* genes in Vhembe District, South Africa. *Am J Trop Med Hyg* 2008;78:577-85.