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ORIGINAL ARTICLE

Rapid Simultaneous Molecular Stool-Based Detection of Toxigenic *Clostridioides difficile* by Quantitative TaqMan Real-Time PCR Assay

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SUMMARY

Background: ***Clostridioides difficile*** **is a major cause of nosocomial infectious diarrhea in hospitalized patients throughout the world.**
Methods: **A multiplex real-time PCR assay was developed and evaluated in comparison with toxigenic culture (TC) (as gold standard method) for direct detection of toxigenic *C. difficile* in fecal specimens. The multiplex real-time PCR assay simultaneously detected glutamate dehydrogenase (*gluD*), toxin A (*tcdA*), toxin B (*tcdB*), and binary toxin (*cdtB*) genes in stool samples.**
Results: **The results of multiplex real-time PCR were compared to those of the TC method in 250 patients suspected of *C. difficile* infection. The prevalence of positive TC was 13.6%. Forty-two stool samples (16.8%) were determined to be *gluD*$^+$ using multiplex real-time PCR. These included 35 (83.3%) toxigenic (32 *tcdA*$^+$, *tcdB*$^+$ and three *tcdB*$^+$) and 7 (20.0%) were *cdtB*$^+$. The multiplex real-time PCR assay had a sensitivity of 91.45%, specificity of 99.54%, and positive and negative predictive values of 97% and 98.6%, respectively, compared to the TC method for diagnosis of *C. difficile*. The analytical sensitivity of the multiplex real-time PCR assay was estimated to be 10^2 CFU/g of stools and 0.0200 pg of genomic DNA from culture. The analytical specificity was determined to be 100% by using enteric and non-*C. difficile* standard bacterial strains.**
Conclusions: **The molecular method developed in the study was rapid, sensitive, and specific for detection of toxigenic *C. difficile*. It is applicable to be performed in clinical laboratories and correlated well with the results obtained by TC.**
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INTRODUCTION

Clostridioides (previously *Clostridium*) *difficile* is a gram-positive rod, sporogenic, obligate anaerobic bacterium, currently known as one of the most important in

and out of hospital and healthcare-associated pathogens in both developed and developing countries [1-3]. It is the main causative agent of antibiotic-associated diarrhea (AAD) and pseudomembranous colitis (PMC) worldwide [2,4]. There is an urgent need to control the spread of this infection in healthcare centers. In addition, this pathogen can be carried asymptomatically in hospitalized patients and healthy adults [5,6]. The high rate of healthy carriers among hospitalized patients along with the presence of patients under antibiotic treatment explains the high prevalence of *C. difficile* associated nosocomial diarrhea [6]. Factors involved in the pathogenesis of *C. difficile* are genes *tcdA* and *tcdB* that code for toxin A (enterotoxin) and toxin B (cytotoxin) which are located in a 19.6 kb chromosomal region called the pathogenicity locus (PaLoc) and those causing *C. difficile*-associated disease (CDAD) including AAD, ileus, bowel perforation, PMC, and toxic mega colon that can be lethal [7,8]. Beside toxins A and B, the bacterium produces a number of other putative virulence factors including a *Clostridioides difficile* binary toxin (*CDT*) (third toxin), fimbriae, SlpA S-layer, fibronectin binding protein (Fbp)A, Cwp84 cysteine protease, putative Cwp66 and CwpV adhesins, and the paracresol [9]. The CDT operon, expressed from the *cdtA* (enzymatic subunit) and *cdtB* (binding subunit), is located outside the PaLoc, at the *CDT* locus in the genome [7]. The genes *cdtA* and *cdtB* together produce an actin-specific ADP-ribosyltransferase as CDT or binary toxin that induces damage to the actin skeleton, leading to cytopathic effects in cell lines [1,8]. Several laboratory methods have been used for diagnosis of *Clostridium difficile* infection (CDI), including enzyme immunoassays (EIAs) for toxins A and/or B, EIAs for glutamate dehydrogenase (GDH), cell cytotoxin neutralization assay (CCNA), toxigenic culture (TC), immunochromogenic assay, and real-time PCR methods [10]. The gold-standard for diagnosis of CDI includes the TC and CCNA methods [11]. These methods are time-consuming, laborious, and difficult to perform (must be performed by trained personnel), and are often less sensitive than molecular assays [10,11]. Enzyme immunoassays (EIAs) for the detection of toxins A and B are rapid, easy to perform, and inexpensive. These EIA assays are more commonly used than the reference standard assays, but their sensitivity and specificity (30% to 70%) has a less than optimal range compared to the gold standard methods [12].

The diagnosis of CDAD is usually based on a clinical history of recent antibiotic(s) usage and diarrhea in combination with laboratory tests [13,14]. Therefore, a new fast, sensitive, and specific method for identification of CDI is needed which can be useful for prescription of effective treatment. Molecular based stool assays were introduced for detection of toxin coding genes of *C. difficile,* such as commercial and in-house-developed real-time PCR. Due to the high sensitivity, specificity, and low turnaround time of these molecular assays, diagnosis and management of CDI has been improved [15]. For this purpose, we developed a multiplex real-time PCR assay for rapid detection of toxigenic *C. difficile* strains in stool samples which is based on the amplification of genes encoding the clostridial toxins, *tcdA* and *tcdB*, and the binding subunit of the binary toxin, *cdtB*. The characteristics of this real-time PCR assay were compared with those of the TC method (as gold standard).

MATERIALS AND METHODS

Patients and specimen processing

From November 2016 to November 2017, a total of 250 unformed (n = 71) or liquid (n = 179) stool specimens collected from hospitalized adults suspected of CDI (90 females and 160 males with age range of 50 to 87 years; mean, 59 years) were studied. All patients were admitted to different wards at three hospitals; Firouzabadi, Firouzgar, and Rasoul Akram in Tehran, Iran. Each patient completed a questionnaire containing different clinical and personal data regarding clinical symptoms, antibiotic usages, and underlying conditions. All stool specimens were stored at 4°C following collection time. For extraction of bacterial DNA, 200 µg of each stool sample (in case of liquid stool samples, 200 µL was added into a bead tube) was measured by digital scale and prepared for the next step. Total microbial DNA was extracted from all stool specimens using FavorPrep™ Stool DNA Isolation Mini Kit (Favorgen Biotech Corp, Taiwan) according to the manufacturer's protocol. DNA purity, quality, and quantity was measured by absorbance spectrophotometry (Nanodrop-1000; NanoDrop Technologies, Wilmington, DE, USA) and agarose gel-electrophoresis. Whole extracted DNAs were immediately stored at -20°C. The stool samples were tested by multiplex real-time PCR and TC (reference method). The multiplex real-time PCR assay was performed within 24 hours after collection. The stool specimens were also cultured for *C. difficile* within 24 hours after collection and tested for TC assay. This project was approved by the Iran University Human Ethics committee (Ethical code: IR.IUMS.FMD.REC 1394.26960).

Primer and probe design

The oligo analysis and design program (Oligoware 3.0) were used for designing the *gluD* (species-specific internal fragment of the glutamate dehydrogenase housekeeping gene), *tcdA, tcdB,* and *cdtB* primers and TaqMan probe sequences (Table 1).

A basic local alignment search tool (BLAST) was performed on these primers and probes in order to evaluate sequences and test specificity of the primers and TaqMan probes. The primers and probes were synthesized by TAG Copenhagen A/S (Copenhagen, Denmark).

Multiplex real-time PCR assay

Multiplex real-time PCR was designed for simultaneous detection of *gluD*, *tcdA*, *tcdB*, and *cdtB* directly from stool samples and was carried out in triplicate. The reaction mixture, in a total volume of 20 µL, contained 0.6 µL of each forward and reverse primer, 0.15 µL of each TaqMan probe, 11 µL of Probe Ex Taq Master Mix (Solis BioDyne, Estonia), and 3.6 µL of template DNA. A 4-plex real time PCR assay was performed by the following cycle conditions: an initial holding at 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing and extension at 60°C for 60 seconds. Also, negative controls including all the elements of the reaction mixture except template DNA were performed in every analysis and no amplified DNA product was ever detected. The reaction was run on a Rotor-Gene 6000 real-time PCR cycler (Qiagen Corbett, Germany).

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

Analytical sensitivity

Two different methods were used for determination of analytical sensitivity of multiplex-real time PCR assay [10]. First, DNA was extracted from the strain of *C. difficile* CD196 (BI/NAP1/027) toxigenic strain and a serial 10-fold dilution (DNA concentrations ranging from 2,000 pg to 0.002 pg) was prepared. Second, a bacterial suspension, approximately 10^2 to 10^9 colony forming units (CFU)/mL, was obtained from an overnight culture of the of *C. difficile* CD196 (BI/NAP1/027) toxigenic strain. Suspension aliquots (0.1 mL) were transferred into 0.9 mL *C. difficile*-negative stool samples (specimens initially reported as negative by real-time PCR). DNA was extracted from bacteria isolated from the stool samples at concentrations ranging from 10^1 to 10^8 CFU/g and was used to evaluate the analytical sensitivity of the multiplex real-time PCR assay [16]. The cycle of quantification (Cq) value for each dilution was recorded. The lowest concentration of DNA obtained from the spiked stool and pure clinical strain of *C. difficile* samples was identified for the analytical sensitivity of our assay. The PCR efficiency for each assay was determined from the slopes of standard curves (E = 2[-1/slope]). Tests were used to compare sensitivity, specificity, and positive and negative predictive values of the multiplex real-time PCR assay.

Analytical specificity

For this purpose, 15 bacterial standard strains including *Bacteroides fragilis* ATCC 23745; *Campylobacter jejuni* ATCC 33291; *Escherichia coli* ATCC 35218, ATCC 25922, *Staphylococcus aureus* ATCC 29213; *Clostridium perfringens* ATCC 13124; *Clostridium sordelii* ATCC 9714; *Enterococcus faecalis* ATCC 29212; *Klebsiella pneumoniae* ATCC 700603; *Proteus mirabilis* ATCC 35659; *Pseudomonas aeruginosa* ATCC 27853; *Salmonella enterica* serovar Typhimurium ATCC 14028; *Enterobacter aerogenes* ATCC 13048; *Yersinia enterocolitica* ATCC 9610; *Shigella flexneri* ATCC 12022 were used for analyzing specificity. Each standard organism was cultured and DNA was extracted via FavorPrep Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp, Taiwan) in accordance with the manufacturer's instructions. DNA purity and quantity were measured by absorbance spectrophotometry (Nanodrop-1000; NanoDrop Technologies, Wilmington, DE, USA). Specificity of the qPCR assay was tested by using 1 ng of genomic DNA extracted from the 15 non-*C. difficile* strains.

Toxigenic culture assay

The stool samples were directly cultured onto anaerobic agar plates (CCFA: cycloserine-cefoxitin-fructose agar) (Himedia Laboratories Pvt. Ltd, India), supplemented with 10% defibrinated sheep blood and selective components (8 mg/L cefoxitin and 250 mg/L cycloserine) following alcohol shock for 1 hour at room temperature. The plates were incubated anaerobically (Whitley Jar Gassing System, UK) at 37°C for up to 5 days and examined daily for growth. Bacterium was identified on the basis of suspected colonies of all *C. difficile* isolates (characteristic morphology; circular yellow colonies or gray-white colonies with raised centers and irregular filamentous or opaque edges, Gram staining, and typical odor; horse barn) and followed a negative indole reaction, yellow-green fluorescence with the Wood's lamp (254 nm), and confirmed by a positive Pro-disk test (L-proline aminopeptidase) reaction [16,17]. The isolates were sub-cultured into Brucella blood agar plates (Merck, Germany) and were incubated anaerobically at 37°C for up to 72 hours. Three or six colonies of *C. difficile* grown on Brucella blood agar plates were inoculated into BHI broth and incubated anaerobically for 5 days. The *C. difficile* isolates were grown in BHI broths and centrifuged for 10 minutes at 4,000 x g. Supernatants were filtered (0.22-µm pore size) and used for determination of toxins. Filtrated supernatants with two dilutions, 1:2 and 1:10, were added in triplicate onto VERO cell monolayers (96-well microtiter plate; SPL life sciences, Korea), followed by incubation for 48 hours at 35°C in 5% CO_2 and then examined using an inverted microscope after 24 and 48 hours for cytopathic effect (CPE) characteristic of *C. difficile* toxins. A positive result was defined as the presence of CPE in at least 50% of the cell monolayer [16,17].

RESULTS

Specificity and analytical sensitivity

Sensitivity analysis revealed that the molecular assay efficiently detected conserved genes as well as toxin genes of the *C. difficile* strain tested. The limit of detection (LoD) of the multiplex real-time PCR was determined to be the same for all targeted genes, at 10^2 CFU/g stool (from spiked stool samples) and ~ 0.0200 pg genomic DNA (from culture genomic DNA

Table 1. Primers and TaqMan probes designed and used in this study for detection of toxigenic *Clostridium difficile.*

Main target	Gene target	Sequences (5´-3´)	Amplicon size (bp)	Gene bank accession number
C. difficile	*gluD*	F: AAAAGATGTAAATGTCTTCGAG R: CCTCTATAACTCTCATAGGTTC Probe: ROX-TTCATAAACTGCTGGTTCCATACCT-BHQ2	116	FN668944 FN668941 FN545816.1 FN665654 FN538970.1 AM180355.1
Toxin A	*tcdA*	F: ATATGAAGTAAGAATTAATAGTGAGG R: TTTACTAGATAAATCGCTCATAATAG Probe: FAM-AAGAACTTCTGGCTCACTCAGGTAA-BHQ1	100	NC_017174.1 FN545816.1 NC_013315.1 CP011968.1 AM180355.1 DQ117264 DQ117248
Toxin B	*tcdB*	F: GCTTCTAAGTCAGATAAATCAG R: TTTATAATACCCTTACTATTAAATGC Probe: HEX-ACTTCTAGTGGTGATGCCTCCATAT-BHQ1	100	FN545816.1 FN538970.1 CP011968.1 AM180355.1 HM062509 HM062509
Binary toxin B	*cdtB*	F: CTCCTGGAGATACTTATCCC R: AATTGATCATAATTTATAGGAATCAG Probe:CY5-CTTTCACCTCTGGCTCTTAACACAA-BBHQ	100	HQ639679.1 HQ639671.1 HQ639673.1 AF271719.1

***gluD* - *Clostridium difficile* specific glutamate dehydrogenase gene, *tcdA* - toxin A gene, *tcdB* - toxin B gene, *cdtB*, binary toxin gene.**

Table 2. Characteristics of multiplex real-time PCR and toxigenic culture (as the reference method).

Assay	Toxigenic culture		Assay performance (95% confidence interval)			
	Negative	Positive	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Multiplex real-time PCR						
Negative	213	1	96.9	98.6	91.4	99.5
Positive	3	33				
Total	216	34				
p-value *			< 0.0001			

PPV - positive predictive value, NPV - negative predictive value. * - p-value for sensitivity, specificity, PPV, and NPV of toxigenic culture and real-time PCR assays.

extraction) (Figure 1). Also, a total of 15 non-*C. difficile* bacteria described previously were tested which showed no cross-reactivity or no amplification signal, thereby demonstrating the specificity of the real-time PCR assay. The reaction efficiencies for *gluD*, *tcdA*, *tcdB*, and *cdtB* PCR were found to be 101%, 98%, 102%, and 102%, respectively, with correlation coefficients of > 0.99.

Clinical performance of *C. difficile* multiplex real-time PCR

A total of 250 fecal samples were used to evaluate the applicability and efficiency of the multiplex real-time PCR assay for identifying *C. difficile* in patients suspected of CDI. All of the stool samples collected were either unformed or of liquid consistency, as recommended for *C. difficile* testing [4]. Primers were design-

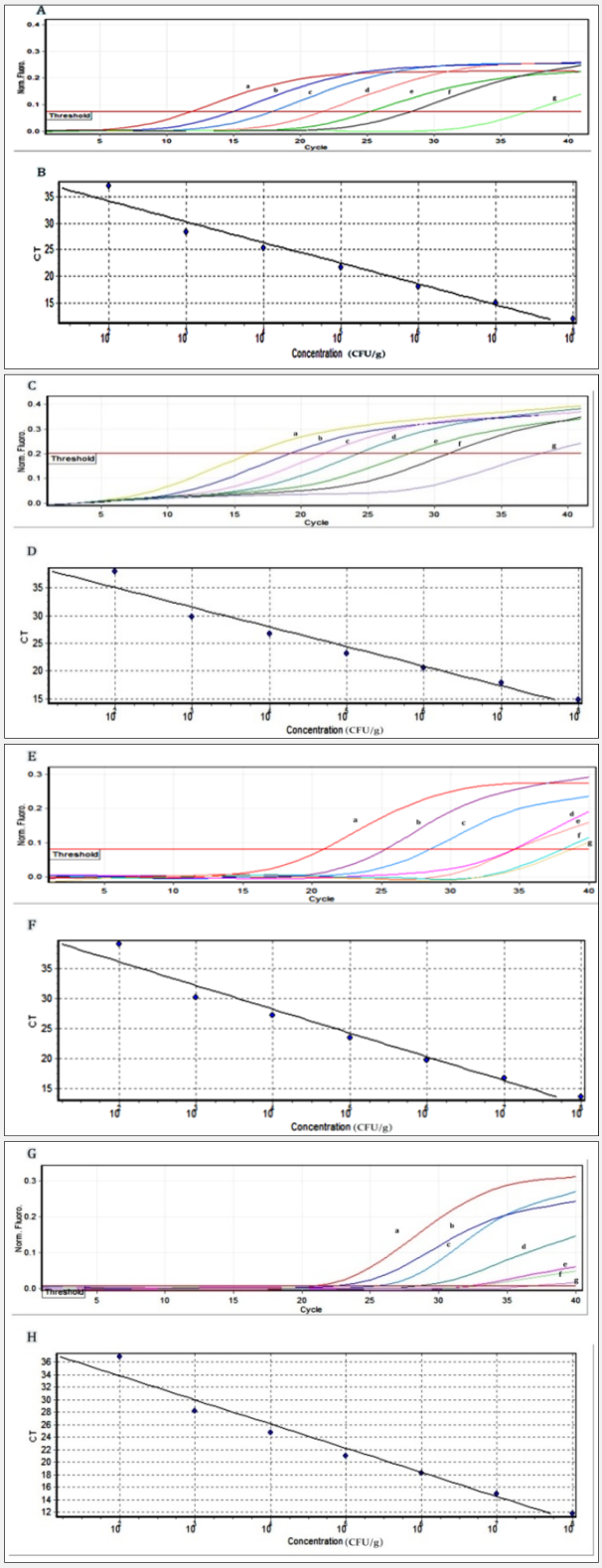

Figure 1. Amplification curves and dilution end-point standard curves of log genome equivalents versus threshold cycle number; quantity of bacteria; (a) 10^8 CFU/g, (b) 10^7 CFU/g, (c) 10^6 CFU/g, (d) 10^5 CFU/g, (e) 10^4 CFU/g, (f) 10^3 CFU/g, and (g) 10^2 CFU/g. The analytical sensitivity of this assay for gluD (A - B), tcdA (C - D), tcdB (E - F), and cdtB (G - H) was approximately 10^2 CFU/g. CFU, colony forming unit.

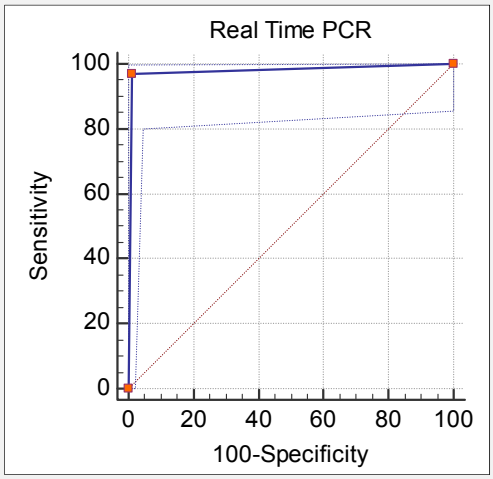

Figure 2. Receiver Operating Curve (ROC) characteristics for the multiplex real-time PCR. AU ROC = area under the ROC curve.

ed and chosen to cover all genetic variants present in GenBank. The 4-plex real time PCR detected all four different toxin gene profiles: 25 *tcdA*$^+$, *tcdB*$^+$, 3 *tcdB*$^+$ and 7 *tcdA*$^+$, *tcdB*$^+$, *cdtB*$^+$, and seven non-toxigenic *tcdA*$^-$, *tcdB*$^-$, *cdtB*$^-$. The prevalence of positive multiplex real-time PCR assay and positive TC was 14% (35/250) (32 *tcdA*$^+$/*tcdB*$^+$, and 3 *tcdB*$^+$) and 13.6% (34/250) (32 *tcdA*$^+$/*tcdB*$^+$, and 2 *tcdB*$^+$), respectively.

Receiver operating characteristic (ROC) curves were calculated, and the area under the curve was 0.955 for the multiplex real-time PCR assay, which was statistically significant (p-value < 0.0001) (Figure 2). There were 33 concordantly positive results, and 213 samples were negative by both tests (Table 2). True positives were defined by toxigenic culture as reference standard approach, and true negatives were defined as negative by all methodologies (multiplex real-time PCR and TC). The performance characteristics were shown in Table 2 and include the sensitivity, specificity, and positive and negative predictive values of the multiplex real-time PCR assay compared to the toxigenic culture test as a reference method.

DISCUSSION

Use of appropriate antibiotic therapy is critical to prevent progression of *C. difficile* [4]. Thus, timely and accurate laboratory diagnosis of *C. difficile* can impact decisions regarding antibiotic therapy and infection control measures [18]. Laboratory diagnosis of CDI continues to be challenging. Currently, several diagnostic tools for detection of *C. difficile* in clinical microbiology laboratories are available [4, 19].

Although cell culture-based methods such as cell culture cytotoxicity assay (CCNA) and toxigenic culture are considered as reference methods with high specificity and sensitivity for detection of *C. difficile*, they suffer from poor turnaround time (minimum 2 to 5 days), high-cost, labor-intensive, lack of standardization among laboratories, are generally unavailable outside research settings, and can only detect toxin B [4,19-22]. Enzyme immunoassay (EIA), and glutamate dehydrogenase (GDH) tests can also be used for detection of this pathogen. The low sensitivity and specificity (50% - 70%) of the toxin A/B EIA assay necessitate developing more accurate methods. The GDH test is rapid (15 - 45 minutes), convenient, inexpensive, and sensitive in stool for detection of non-toxigenic *C. difficile*. However, the test cannot differentiate between toxigenic strains and non-toxigenic strains (20% of *C. difficile* strains do not produce toxin) [4,19,23,24]. Nucleic acid amplification methods are increasingly becoming popular for detection of microorganisms in clinical specimens [4,18,25]. PCR-based methods were developed for specific detection of *C. difficile* toxin genes in feces samples; however, some PCR assays are all coupled with time-consuming post-PCR manipulations for analysis of the amplification products, i.e., agarose gel electrophoresis and Southern hybridization [4,18,26-28]. Thus, we developed and evaluated a multiplex real-time PCR assay for rapid, simultaneous detection of toxigenic *C. difficile* strains directly in stool samples which is based on the amplification of genes encoding the clostridial toxins, *tcdA* and *tcdB*, and the binding subunit of the binary toxin, *cdtB*.

More advanced molecular stool-based assays are introduced for detection of *C. difficile* such as commercial and in-house-developed real-time PCR assays with high sensitivity (77.3% - 97.1%), specificity (93% - 100%), fast reaction time (1 - 3 hours), and convenience [10]. The performance characteristics of our multi real-time PCR assay are in agreement with those data, with a sensitivity and a specificity of 91.45% and 99.54%, respectively. It performed better than the cytotoxicity assay when comparing to TC as the gold standard method [29].

One sample tested negative by the multi real-time PCR assay, but cytotoxin was positive by the TC test. This suggests that the concentration of *C. difficile* in the patient stool sample was lower than the analytical sensitivity of our assay. This isolate was positive when its DNA was extracted and used as the template for PCR, suggesting that the false-negative results were not related to mismatch of primers and/or probes [29].

In this study, three samples were negative in the toxigenic culture, but were positive in the real-time PCR assay. This may be due to low number of microorganisms in stool or growth inhibition caused by concomitant anti-*C. difficile* treatment [10].

The analytical sensitivity of our assay was 10^2 CFU/g of stool and 0.02 pg genomic DNA. Results were > 1,000 -fold more sensitive than the conventional PCR assay described by Guilbault et al. [30], and Lotfian et al. [31]; however, it was comparable to other published real-time PCR assays targeting *C. difficile* in stool samples [4,10,29,32-37]. The cycle thresholds ranged from 13 to 39 corresponding to a range of approximately 10^1 to 10^8 CFU/g spiked stool samples, showing that *C. difficile*-infected samples presented a broad range of bacterial loads.

The role of binary toxin in disease is not well established. It may be associated with the hyper-virulent epidemic BI/NAP1/O27 strain which had increased CDI mortality [38,39]. In our study, the binary toxin gene (*cdtB*) was found in 2.8% of the stool samples. This percentage is lower than what was shown previously for selected strains isolated from other studies (13.7%, Spigaglia & Mastrantonio (2002); 6%, Gonclaves et al., (2004); 5.8%, Geric et al., (2004); 4.2%, Arroyo et al., (2006); 8.6 %, Pituch et al., (2005); and 4.5%, Alonso et al., (2005), but similar to the estimated frequencies of Geric et al., (2004), Rupnik et al., (2003), Kim et al., (2010), Samie et al., (2008) with 2.8%, 1.6%, 2%, 3.7% of all *C. difficile* isolates, respectively [39-48].

The incidence of strains *tcdB*$^+$/*tcdA*$^-$ *C. difficile* ranges from 3% to 92% worldwide [10,44,49-53], although the clinical characteristics of these strains have not been well studied. In the current study, three (8.57%) out of 35 toxigenic isolates were *tcdA*$^-$/*tcdB*$^+$. Our assay has certain limitations as it was not designed to detect deletions in the *tcdC* gene, a known marker of hypervirulent strains, and other known virulence genes in the PaLoc (*tcdE* and *tcdR*).

In brief, the ~ 2-hour real-time PCR assay developed in this study can readily detect *C. difficile* toxin genes *tcdA*, *tcdB*, and *cdtB* which is quite promising for direct detection of *C. difficile* from stool samples.

CONCLUSION

The molecular assay developed in this study had performance characteristics comparable to the TC assay for detection of toxigenic *C. difficile*. In addition, more investigations are needed to determine the LoD for these emerging assays, and also target *tcdC*, *tcdE*, and *tcdR* genes using the above described assays. However, for further confirmation of the results, it may still be necessary/recommended, to couple this assay with other testing modalities.

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Ethical Approval:
This project was approved by the Iran University Human Ethics committee (Ethical code: IR.IUMS.FMD. REC 1394.26960).

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Declaration of Interest:
There are no conflicts of interest.

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