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RESEARCH ARTICLE

Simultaneous detection and characterization of toxigenic *Clostridium difficile* directly from clinical stool specimens

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Abstract **We employed a multiplex polymerase chain reaction (PCR) coupled with capillary electrophoresis (mPCR-CE) targeting six *Clostridium difficile* genes, including *tpi*, *tcdA*, *tcdB*, *cdtA*, *cdtB*, and a deletion in *tcdC* for simultaneous detection and characterization of toxigenic *C. difficile* directly from fecal specimens. The mPCR-CE had a limit of detection of 10 colony-forming units per reaction with no cross-reactions with other related bacterial genes. Clinical validation was performed on 354 consecutively collected stool specimens from patients with suspected *C. difficile* infection and 45 isolates. The results were compared with a reference standard combined with BD MAX Cdiff, real-time cell analysis assay (RTCA), and mPCR-CE. The toxigenic *C. difficile* species were detected in 36 isolates and 45 stool specimens by the mPCR-CE, which provided a positive rate of 20.3% (81/399). The mPCR-CE had a specificity of 97.2% and a sensitivity of 96.0%, which was higher than RTCA ($x^2 = 5.67$, $P = 0.017$) but lower than BD MAX Cdiff ($P = 0.245$). Among the 45 strains, 44 (97.8%) were determined as non-ribotype 027 by the mPCR-CE, which was fully agreed with PCR ribotyping. Even though ribotypes 017 ($n = 8$, 17.8%), 001 ($n = 6$, 13.3%), and 012 ($n = 7$, 15.6%) were predominant in this region, ribotype 027 was an important genotype monitored routinely. The mPCR-CE provided an alternative diagnosis tool for the simultaneous detection of toxigenic *C. difficile* in stool and potentially differentiated between RT027 and non-RT027.**

Keywords *Clostridium difficile*; multiplex PCR; capillary electrophoresis; detection; characterization

Introduction

Clostridium difficile is a spore-forming anaerobic bacterium colonizing the large intestine as a part of normal microbiota [1]. Toxigenic *C. difficile* can cause antibiotic-associated diarrheal disease in patients after hospitalization and/or antibiotic treatment [2]. *C. difficile* infection (CDI) has also been recognized as a cause of pseudomembranous colitis and *C. difficile*-associated diarrhea since 1977 [3]. After the emergence of hypervirulent ribotype 027 strains, the incidence, complications, morbidity, and mortality of CDI have increased dramatically in the past decade [4,5], and the outbreaks of CDI have been described in several countries [5–9]. Therefore, clinical diagnosis and surveillance for CDI are routinely performed in the United States and Europe [10,11].

CDI-related diseases are mediated by *C. difficile* toxins. *C. difficile* produces two high-molecular weight toxins, the enterotoxin A (*tcdA*) and the cytotoxin B (*tcdB*), with its coding genes integrated with two regulatory genes (*tcdC* and *tcdD*) and a putative holin gene (*tcdE*), forming a 19.6 kb chromosomal region that named the pathogenicity locus (PaLoc) [12,13]. A third toxin, an actin-specific ADP-ribosyltransferase and encoded by *C. difficile cdtA* and *cdtB* genes, is located on the locus of the chromosome separated from the PaLoc. *CdtA* and *cdtB* subunits form the binary toxin [14]. The triose phosphate isomerase (*tpi*), a housekeeping gene with high specificity for *C. difficile*, has been used for the identification of *C. difficile* in other assays [15,16]. Meanwhile, an 18 base pairs deletion in

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tcdC gene has been recognized as a typical gene marker for the identification of epidemic hypervirulent ribotype 027 strains [4,17].

A large number of methods have been available for the laboratory diagnosis of CDI in the past decade. The toxigenic culture assay was recognized as a gold standard for the identification of toxigenic *C. difficile*. However, this method was time consuming (2–3 days), labor-intensive, and not suitable for wide use in clinical microbiology laboratories [18,19]. Enzyme immunoassays (EIAs) have been adopted for the direct detection of toxins A and/or B, glutamate dehydrogenase (GDH) of *C. difficile* in stool [20]. However, toxin A/B detection based on EIA is no longer recommended as a primary test due to poor sensitivity, and GDH detection cannot differentiate toxigenic *C. difficile* from nontoxigenic *C. difficile* [18,21]. The molecular assays targeting *tcdA*, *tcdB*, and/or *tcdC* gene have shown promising performance in recent years. By far, a total of eight commercial kits have been cleared by the Food and Drug Administration in the United States [15,18–20,22–24]. Some advanced techniques also have been reported for detecting toxigenic *C. difficile* with satisfactory capability [19,25,26]. However, molecular tests have been indicated too sensitive and have led to overdiagnosis for CDI [27]. Most current molecular assays generally target one gene without any identification/characterization capacity (except for the Cepheid assay) [28]. Therefore, simultaneous detection of multiple genes in the diagnosis of *C.difficile* might facilitate clinical diagnosis. Moreover, the high cost might be also one of factors that hinders wider usage in clinical setting in developing countries.

CDI has been reported in several cities in China, including two cities which recently revealed ribotype 027 [9,29–31]. CDI has become a main reason of diarrhea-related diseases in Chinese inpatients. Moreover, the morbidity of CDI may be underestimated because of the lack of data reported. Consequently, demanding for clinician to start CDI testing for patients with diarrhea is urgent because CDI cases increase gradually in recent years. In addition, monitoring CDI outbreak and controlling the nosocomial spread of infection in China should be performed by CDC. Nevertheless, only three commercial kits (Mini-VIDAS, BioMerieux, France; GeneXpert, Cepheid, USA; QUIK CHEK complete and A/B II ELISA, TechLab, USA) were approved by the China Food and Drug Administration (CFDA). Thus, the popularization and application of CDI diagnosis might be difficult.

A multiplex PCR coupled with capillary electrophoresis (mPCR-CE) technology has been utilized for the identification of seven foodborne pathogens and discrimination between pathogenic and non-pathogenic *Escherichia coli*, genetically modified organism, and deletion or duplication genotype [32–34]. In this study, we developed and evaluated a mPCR-CE assay for simultaneous detection of six *C. difficile*-specific genes and mutations. The clinical validation of the mPCR-CE was performed on a panel of well characterized *C. difficile* strains and stool specimens collected from patients with diarrhea.

Materials and methods

Bacterial strains and specimen collection

All standard bacterial strains were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) (Table 1). PCR ribotypes were provided at the ATCC website, and the ATCC BAA-1870 strain is PCR ribotype 027. All strains were cultured by the standard microbiological procedures as previously described [35]. Clinical stool specimens from patients with suspected CDI were consecutively collected between August 13 and December 21, 2013 at three hospitals: Department of Neurosurgery, First People's Hospital of Xiaoshan District; Department of Medical Oncology, First Affiliated Hospital of Zhejiang University College of Medicine; and Department of Gastroenterology, Hangzhou First People's Hospital in Hangzhou, Zhejiang. Liquid, soft, or semi-solid stool specimens with sufficient volumes were stored at −80 °C and transported to the Zhejiang Provincial Center for Disease Control and Prevention (ZJCDC) within 72 h for further testing. Aliquots were made for each stool specimen and were tested by the mPCR-CE assay, BD MAX Cdiff assay, and real-time cell analysis assay (RTCA) within 48 h after collection (see below). The stool specimens were cultured for *C. difficile* using cefoxitin-cycloserine fructose agar plates (UK BioTech Inc., Hangzhou, Zhejiang, China) according to our previous reports [36]. This study as a part of other research was approved by the Institutional Review Boards of ZJCDC.

mPCR-CE assay

The genomic DNA of the *C. difficile* strains was extracted by using the QIAamp DNA mini kit (Cat No. 51304) (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions [9]. Genomic DNA was extracted from stool specimens by using a MOKO DNA stool kit (MOKObio Biotechnology R&D Center Inc., Durham, NC, USA) per manufacturer's instructions. A positive control was prepared by diluting *C. difficile* BAA-1870 (PCR ribotype 027) 1:10 into pooled *C. difficile*-negative stool specimens according to the method previously reported [37]. Serial concentrations of the dilutions ranged from 9.0×10^7 to 9.0×10^1 colony-forming units (CFU) per gram of stool specimens.

Six primer sets were designed to detect 5 *C. difficile*-specific genes (*tcdA*, *tcdB*, *cdtA*, *cdtB*, and *tpi*) and 18 base

Table 1 Bacterial strains

Bacterial origin	ATCC number	*tcdA*	*tcdB*	*cdtA*	*cdtB*	18 bp deletion in *tcdC*
C. difficile	43255	+	+	−	−	WT[b]
C. difficile	700057	−	−	−	−	WT
C. difficile	BAA-1870	+	+	+	+	18 bp deletion
C. difficile	BAA-1804	+	+	−	−	WT
C. difficile	BAA-1803	+	+	+	+	18 bp deletion
C. difficile	BAA-1801	−	−	−	−	WT
C. difficile	43598	+	+	−	−	WT
C. difficile	BAA-1812	+	+	−	−	WT
Vibrio cholerae	BAA-2163, 39541, 25870, 55866	N/A[a]				
Vibrio parahemolyticus	43996	N/A				
Vibrio vulnificus	27562	N/A				
Clostridium perfringens	27057	N/A				
Clostridium botulinum	19397	N/A				
Campylobacter jejuni	33560	N/A				
Enterobacter aerogenes	13048	N/A				
Escherichia coli O157:H7	43888	N/A				
Listeria monocytogenes	19111	N/A				
Shigella flexneri	55556	N/A				
Salmonella enterica	51960	N/A				

[a]N/A: not applicable. [b]WT: wild type with no 18 bp deletion.

pairs deletion in *tcdC* gene. All the primer pairs were designed by using Primer Premier 5.00 software, as shown in Table 2. All the forward primers were labeled at the 5′ end with carboxyfluorescein (FAM). Amplification was performed in a 25 μL reaction consisting of 1 × HotStar Taq Master Mix (Qiagen, Inc., Valencia, CA, USA), five primer sets, and 1 μL extracted DNA. Amplification was carried out in an Eppendorf AG PCR instrument with a 10 min initial enzyme activation at 95 °C, and DNA was amplified for 30 cycles consisting of 20 s at 95 °C, 20 s at 58 °C, and 25 s at 72 °C, followed by a 5 min final elongation step at 72 °C. PCR fragments were analyzed in an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA) as described previously [38]. Every test run included a positive control, a negative control, and a blank control.

RTCA

RTCA assay uses a real-time cell analysis system (xCELLigence, ACEA Biosciences, San Diego, CA, USA) to detect *C. difficile* toxin B. 5% (wt/vol, vol/vol) of stool specimen was diluted in cold Hanks buffered salt solution and then centrifuged. Supernatant was collected after centrifugation, and toxin level was tested quantitatively on an xCELLigence RTCA system as previously described [19].

BD MAX

The BD MAX Cdiff assay was performed according to the manufacturer's instructions as previously described. About 10 μL of stool sample was added to the BD MAX sample buffer, mixed completely, and vortexed for 1 min. DNA extraction was done by using a magnetic-bead technology with a sample-processing control. Afterward, 4.2 μL of the PCR mixture was well amplified in the cartridge. The results were exported using the BD MAX system software. The *tcdC* gene was directly sequenced as in the previous studies [17,39].

Hands-on time, test turnaround time, and cost per test

Hands-on time and test turnaround time were calculated according to 16 samples. Then, the time of pretreatment, including cell seeding and growing up to the plateau, was excluded for the RTCA assay. The cost of BD MAX Cdiff was a reference price of the kit that we purchased. The cost of the RTCA assay was approximately calculated according to our previous studies [19,40].

PCR ribotyping and sequencing

PCR ribotyping was performed by using a capillary gel electrophoresis with primer pairs as described previously [9,41,42]. After the PCR reaction was completed, the fragments were analyzed with an ABI 3100 genetic analyzer (Applied Biosystems, USA) with 36 cm capillary loaded with a POP4 gel (Applied Biosystems). After the 1 μL PCR fragment was mixed with 10 μL Hi-Di™ formamide (Applied Biosystems) and 0.5 μL AGCU SIZ *680* size standard (AGCU ScienTech Inc., Wuxi, Jiangsu,

Table 2 Primer pairs

Gene target	Primer name	Sequence (5′–3′)	Primer concentration (μmol/L)	Product size (bp)
Tpi	*tpi*-F	AAAGAAGCTACTAAGGGTACAAA	0.80	229
	tpi-R	CATAATATTGGGTCTATTCCTAC	0.80	
tcdA	*tcdA*-F	AGGGCTAATAATCTTACTATGTC	0.60	258
	tcdA-R	ATCTCAAATCAATAAACCTACAG	0.60	
tcdB	*tcdB*-F	AATGCATTTTTGATAAACACATTG	0.50	329
	tcdB-R	AAGTTTCTAACATCATTTCCAC	0.50	
tcdC	*tcdC*-F	TGCTGAACCATGGTTCAA	1.0	177/159
	tcdC-R	GCTAATTGGTCATAAGTAATAC	1.0	
cdtA	*cdtA*-F	TTACCTAGAAATACTGGTATGTTA	1.1	303
	cdtA-R	AATTATTAATTGCAGTATATCCTC	1.1	
cdtB	*cdtB*-F	CTACAAGATAAAAATTTAGGTTCA	0.85	361
	cdtB-R	CTGTATATGGATCTCCAGCA	0.85	

China), the mixture was injected into a capillary at 3 kV for over 10 s. The total running time was about 30 min at a run voltage of 15 kV. The size of each peak was determined by Genemapper ID-X software 1.3 (Applied Biosystems). The capillary-sequencer-based PCR-ribotyping data were analyzed at WEBRIBO website (https://webribo.ages.at/) [42].

Data analysis

The mPCR-CE results were categorized into (1) no *C. difficile*, (2) nontoxigenic *C. difficile*, (3) toxigenic *C. difficile*, and (4) toxigenic, ribotype 027 *C. difficile*. The result was considered invalid if any toxin genes were present while the species-specific *tpi* gene was absent (Table 3). A combined reference standard was defined as concordant results for two or more of the following assays: mPCR-CE, BD MAX Cdiff, and RTCA. For analytical specificity and sensitivity determinations, the known concentrations of toxigenic *C. difficile* strains with *tcdA*/*tcdB* and/or *cdtA/cdtB* genes, *tcdC* gene with 18 bp deletion, and nontoxigenic *C. difficile*, *C. botulinum*, *C. perfringens*, and other enterobacteria were evaluated. The clinical sensitivity, specificity, and positive and negative predictive values (PPV and NPV, respectively) of the mPCR-CE assay were determined. Test turnaround time, hands-on time, and cost of reagents were calculated. Odds ratios (OR), 95% confidence interval (CI), and *P* values were calculated by using the SPSS version 20.0 software (SPSS Inc., Chicago, IL, USA). The *P* values of $\leqslant 0.05$ were considered statistically significant.

Results

The primer pairs of six target genes were designed for amplifying PCR products with different sizes (Table 2). After the analysis of capillary electrophoresis, different PCR amplicons appeared in the different positions depending on different migration rates. The FAM channel was chosen for collecting fluorescent signals. After the optimization of PCR conditions, including the concentration and ratio of primer pairs, annealing temperature, extension time, and number of PCR cycles, a multiplex PCR coupled with capillary electrophoresis was established to detect *tcdA*, *tcdB*, *cdtA*, *cdtB*, and *tpi* genes, as well as an 18 bp deletion in *tcdC* gene. All the qualitative results from the mPCR-CE are interpreted in Table 3.

To determine the analytical specificity of the mPCR-CE assay, a panel of diarrhea-related pathogens was tested. No peaks corresponded to the target products found in *Clostridium perfringens*, *Clostridium botulinum*, *Vibrio cholerae*, or other diarrhea-related pathogens (Table 1). The specific peak in a 229 bp of *tpi* gene was observed in all *C. difficile* strains. The assay specifically detected at least one toxin gene, including *tcdA*, *tcdB*, *cdtA*, and *cdtB* genes in toxigenic *C. difficile* strains with no cross-reaction with nontoxigenic *C. difficile* and other strains. In addition, the peak positions of four toxin genes were in accordance with their sizes of PCR products. The assay also detected an 18 bp size variation in *tcdC* gene between ribotype 027 and nonribotype 027 (Fig. 1). The analytical sensitivity of the mPCR-CE assay was determined as above mentioned. The corresponding peaks of four genes, including *tcdA*, *tcdB*, *cdtA*, and *cdtB*, and a deletion in *tcdC* gene were observed for purified DNA with a limit of a detection of 10 CFU per reaction as performed on 10-fold diluted *C. difficile* spiked in sterile water. The limit of detection was 9.0×10^4 CFU per gram of stool specimens for *C. difficile* spiked in pooled negative stool specimens.

A total of 354 stool specimens and 45 *C. difficile* isolates were tested to determine the clinical validation of the

Table 3 Interpretation of mPCR-CE detection results in 354 stool specimens

tpi[a]	*tcdA*	*tcdB*	*cdtA*	*cdtB*	18 bp deletion in *tcdC*	Interpretation
−	N/A[b]	N/A	N/A	N/A	N/A	No *C. difficile*
+	−	−	N/A	N/A	N/A	Nontoxigenic *C. difficile*
+	+/−	−/+	−	−	WT[c]	Toxigenic *C. difficile*
+	+	+	−	−	WT	Toxigenic *C. difficile*
+	+	+	+	+	WT	Toxigenic *C. difficile*
					M[d]	Toxigenic and ribotype 027 *C. difficile*
+	+	+	−	+	WT	Toxigenic *C. difficile*
					M	Toxigenic and ribotype 027 *C. difficile*
+	+	+	+	−	WT	Toxigenic *C. difficile*
					M	Toxigenic and ribotype 027 *C. difficile*
+	+	−	+	+	WT	Toxigenic *C. difficile*
					M	Toxigenic and ribotype 027 *C. difficile*
+	+	−	+	−	WT	Toxigenic *C. difficile*
					M	Toxigenic and ribotype 027 *C. difficile*
+	+	−	−	+	WT	Toxigenic *C. difficile*
					M	Toxigenic and ribotype 027 *C. difficile*
+	−	+	+	+	WT	Toxigenic *C. difficile*
					M	Toxigenic and ribotype 027 *C. difficile*
+	−	+	+	−	WT	Toxigenic *C. difficile*
					M	Toxigenic and ribotype 027 *C. difficile*
+	−	+	−	+	WT	Toxigenic *C. difficile*
					M	Toxigenic and ribotype 027 *C. difficile*
−	At least one toxin gene positive				WT	Invalid results
					M	

[a]+, positive; −, negative. [b]N/A, not applicable. [c]WT, wild type with no 18 bp deletion. [d]M, mutant type with 18 bp deletion.

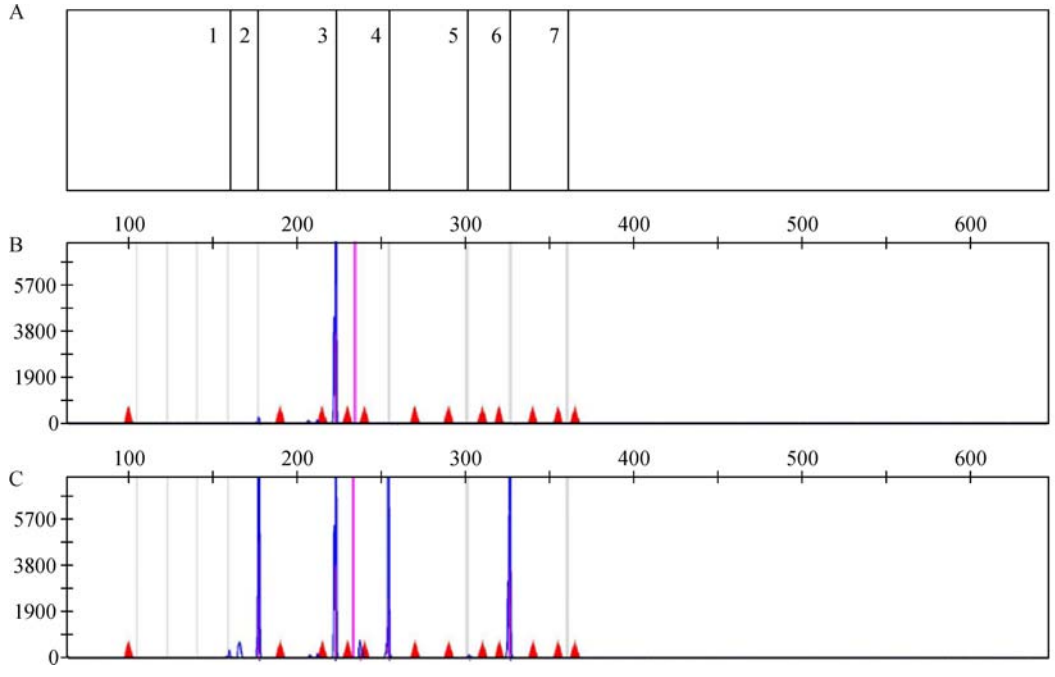

Fig. 1 Representative mPCR-CE results in clinical specimens. Blue peaks, red triangles, *X* axis, and *Y* axis represent positive PCR products, standard size markers, length of PCR amplicon, and fluorescence intensity, respectively. (A) A schematic diagram of distribution of six target *C. difficile* genes according to their sizes. 1: Presence of an 18 bp deletion in *tcdC* gene. 2: Absence of an 18 bp deletion in *tcdC* gene. 3: *tpi* gene. 4: *tcdA* gene. 5: *cdtA* gene. 6: *tcdB* gene. 7: *cdtB* gene. (B) *tpi* gene was positive, indicating that no *C. difficile* existed in this clinical stool specimen. (C) This clinical stool specimen was positive for toxigenic *C. difficile* with *tcdA*, *tcdB*, and *tpi* genes without an 18 bp deletion in *tcdC* gene.

mPCR-CE assay. The results were determined by the combined reference standard. The mPCR-CE assay identified toxigenic *C. difficile* in 36 (80.0%) isolates giving the sensitivity and specificity of 94.6% and 87.5%. The mPCR-CE assay identified toxigenic *C. difficile* in 45 (12.7%) stool specimens with the sensitivity, specificity, PPV, and NPV of 97.4%, 97.5%, 82.2%, and 99.7%, respectively. The sensitivity of the mPCR-CE assay (96.0%) for toxigenic *C. difficile* detection was higher than that of the RTCA assay (82.7%, OR = 5.03, 95% CI = 1.37–18.47, $P = 0.017$) but lower than that of the BD MAX (100%, Fisher exact $P = 0.245$). By contrast, the specificity (97.2%; OR = 1.12, 95% CI = 0.68–1.82, $P = 0.820$) of the mPCR-CE was higher than those of the BD MAX Cdiff (Table 4).

A total of 18 PCR ribotypes were found in the 45 strains, as shown in Fig. 2. No ribotype 027 was detected by either mPCR-CE or PCR ribotyping in stool specimens. Furthermore, no $cdtA^+cdtB^-$ and $cdtA^-cdtB^+$ strains were found. PCR ribotypes 017 (n = 8, 17.8%), 001 (n = 6, 13.3%), and 012 (n = 7, 15.6%) were predominant in this region, whereas the other PCR ribotypes included 020, 085, 039, 049, and so on. We compared the results of the mPCR-CE with that of PCR ribotyping for ribotype 027 in 45 strains. Among these strains, 44 (97.8%) were confirmed as nonribotype 027 strain by the mPCR-CE, which was in accordance with the results of PCR ribotyping.

Values representing hands-on time, test turnaround time, and cost per test for the three assays are compared in Table 5. The mPCR-CE had shorter hands-on time (25 min) than that of the RTCA (55 min) but longer than that of the BD MAX Cdiff (8 min). However, the test turnaround of the mPCR-CE was similar to that of the BD MAX Cdiff. Notably, the cost of the mPCR-CE was extremely cheaper than those of the two assays.

Discussion

CDI has become a serious public health-related issue worldwide as the leading cause of antibiotic-associated diarrhea, resulting in a heavy burden to global healthcare systems [43]. The emergence of PCR ribotype 027 caused a tremendous change in the global molecular epidemiology of *C. difficile* and the clinical outcomes of CDI [44]. Numerous studies indicated that CDI cases have been increasing in China [36,45–48]. Moreover, clinical cases have been related with ribotype 027 in China [30,49]. Hence, carrying out toxigenic *C. difficile* testing in clinical laboratories and centers for disease control and prevention in China is necessary. In this study, we reported for the first time the use of mPCR-CE assay for the qualitative detection of six *C. difficile*-specific genes. The following information was reported as follows: the presence or absence of toxigenic or nontoxigenic *C. difficile* and whether or not *C. difficile* strain is detected indicate PCR ribotype 027.

Molecular methods and kits based on various technologies, including real-time PCR, multiplex PCR, loop-mediated isothermal amplification, and DNA microarray, have been existing [37,50–52]. Among these assays, the GeneXpert Epi and the Verigene Cdiff assays were commercial kits available to detect toxigenic *C. difficile* and simultaneous differentiation between ribotype 027 and non ribotype 027 based on deletions in *tcdC* gene, with promising performance [43,53]. In our study, nontoxigenic isolates were distinguished from toxigenic ones using the

Table 4 Sensitivity, specificity, and predictive values for toxigenic *C. difficile* by mPCR-CE[a], BD MAX, and RTCA assays

Type	Assay	Number of stool specimens with indicated result				Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
		$S^+ T^+$	$S^+ T^-$	$S^- T^+$	$S^- T^-$				
Stool	mPCR-CE	37	1	8	308	97.4	97.5	82.2	99.7
	BD MAX Cdiff	38	0	10	306	100.0	96.8	79.2	100.0
	RTCA	31	7	2	314	81.6	99.4	93.9	97.8
Strain	mPCR-CE	35	2	1	7	94.6	87.5	N.A.	N.A.
	BD MAX Cdiff	37	0	1	7	100.0	87.5	N.A.	N.A.
	RTCA	31	6	0	8	83.8	100.0	N.A.	N.A.
Total	mPCR-CE	72	3	9	315	96.0	97.2	N.A.	N.A.
	BD MAX Cdiff	75	0	11	313	100.0	96.6	N.A.	N.A.
	RTCA	62	13	2	322	82.7	99.4	N.A.	N.A.

[a]Only these results were analyzed according to four toxin genes (*tcdA*, *tcdB*, *cdtA*, and *cdtB*).
S, standard; T, test. N.A., not applicable.

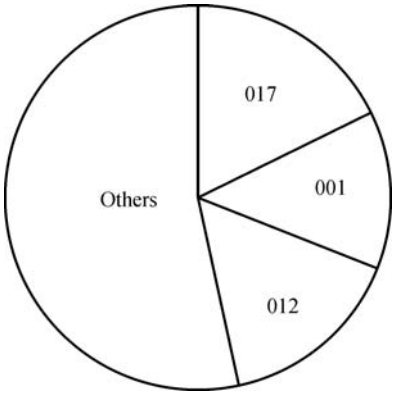

Fig. 2 Distribution of PCR ribotypes.

Table 5 Comparison by the three assays for toxigenic *C. difficile* detection[a]

Assay	Hands-on time (min)	Test turnaround time (min)	Cost per test (RMB)[b]
mPCR-CE	25	132.5	35.5
BD MAX Cdiff	8	130	150
RTCA	55[c]	1381.1	82.5

[a]Hands-on time and test turnaround time were calculated on the basis of a full run of 16 stool specimens.
[b]Cost per test was not an exact cost, because the reagent prices fluctuated continually.
[c]This time was calculated after the cell grew in a stable phase.

mPCR-CE assay. This advantage was meaningful to get the comprehensive information of patients with suspected CDI and avoid the use of antibiotics that easily reduce CDI in patients with nontoxigenic *C. difficile*. This assay presented good analytical sensitivities with 10 CFU per reaction for purified DNA and 9.0×10^4 CFU per gram of pooled *C. difficile*-negative stool specimens, which was 100-fold more sensitive than the PCR assay described previously [54] and similar to the sensitivity of the real-time PCR assays previously reported [37,52]. Furthermore, clinical specimens could be directly detected after rapid and simple DNA extraction by the mPCR-CE assay, which showed a sensitivity of 96.0% and a specificity of 97.2% for strains and stool specimens. The performance of this assay was similar to that of the BD MAX Cdiff assay, a FDA clearance kit, but better than that of the RTCA.

The BD MAX Cdiff assay has been evaluated in previous studies, which has shown more promising performance compared with those of other commercial kits [28,55,56]. However, the BD MAX Cdiff and the CFDA cleared GeneXpert *C. difficile*. Epi kits are very expensive, which hinders their extensive use in clinical laboratories in China. The cost of the mPCR-CE assay evaluated based on the price of all related reagents in this period was approximately four folds less than that of the BD MAX Cdiff with research-use only, which was a reference price of the kit that we purchased (Table 5). In our assay, a rapid stool DNA extraction assay was employed to shorten the hands-on time and turnaround time compared with that of the QIAamp DNA stool kit. Thus, the mPCR-CE had shorter hands-on time than that of the RTCA but similar to that of the BD MAX Cdiff (data not shown). Combining DNA extraction with PCR reaction is possible for automatic result analysis, including GeneXpert and BD MAX platforms. Based on our study, mPCR-CE might be a preferred assay with a promising prospect for clinical diagnosis and molecular epidemiology.

The genetic characteristics of PCR ribotype 027 have been well characterized with binary toxin, 18 bp, and nucleotide 117 position deletions in *tcdC*, which were markers tested by different methods [4,43,57]. Persson *et al*. described an exception in which one isolate was PCR ribotype dk120 having binary toxin genes with 18 bp deletion and without Δ117. This finding made the 18 bp deletion 98% and the Δ117 deletion 100% specific for ribotype 027 [57]. In this study, we also found a *C. difficile* isolate harboring *tcdA*, *tcdB*, binary toxin genes, and 18 bp deletion in *tcdC* gene in which PCR ribotype was AI-56, not 027 (No. GenBank: KF962642.1). The 18 bp deletion and nucleotide 117 position substitution in *tcdC* gene were confirmed by direct sequencing (data not shown). However, the Δ117 deletion has been reported in nonPCR ribotype 027 strains. A total of 97.8% of strains were confirmed as nonribotype 027 strains, which were in accordance with the results of PCR ribotyping and suggesting that 18 bp deletion and Δ117 deletion in *tcdC* gene might be preliminary screening biomarkers but not as absolute markers for the identification of ribotype 027. Hyper-virulent *C. difficile* still needs to be identified by multilocus sequence typing and PCR ribotyping.

Based on the current reports on molecular epidemiology, the molecular characteristics of *C. difficile* strains were extremely different from different regions. Ribotype 017 with truncated *tcd*A may be a potential epidemic strain in China [9,29,46]. The mPCR-CE assay did not detect the deletion in *tcdA* to identify ribotype 017. Moreover, a variety of drug-resistant genes was related to *C. difficile* antibiotics resistance, including *ermB* (MLSB antibiotics resistance gene), *tetM* (tetracycline resistance gene), and *GyrA* or *GyrB* [58–60]. Therefore, we should add more targets to this assay to get more information in one reaction. In addition, limitation occurred on internal controls in this assay. Previous studies showed that the 16S rRNA gene was usually chosen as an internal control in a variety of molecular methods [52,61]. In this study, we amplified genomic DNAs extracted from stool specimens by the 16S rRNA gene primers [62] and analyzed PCR products as internal controls through capillary electrophoresis. Determining the validity of the internal control became difficult because of multiple peaks with different

sizes. The cause of this finding might be a mixture of genomic DNAs from various bacteria in stool specimens and PCR products that contained different amplicons from different species by 16S rRNA universal primers [62,63]. Thus, we should further screen the suitable targets as internal controls in this assay.

Among the 45 strains, three ribotypes (017, 001, and 012) were dominant, whereas others belonged to 15 ribotypes. No significant differences occurred among the three departments (data not shown). These results were similar to the previous reports in Hangzhou and Beijing. Moreover, these patients were described as having community-onset and healthcare facility-associated diseases according to the guidelines of the Society for Healthcare Epidemiology of America and the Infectious Disease Society of America [64]. Thus, we inferred that CDI cases might be sporadic in this regional communities. Even though ribotype 027 was not found in these regions, we should pay more attention to this genotype due to its hyper-virulence property [64]. Continued surveillance is extremely important to monitor the characteristics of *C. difficile* epidemiology in the communities in China by using the mPCR-CE and/or other assays.

In conclusion, we successfully developed mPCR-CE for simultaneous detection of six *C. difficile*-specific genes to discriminate between toxigenic and nontoxigenic *C. difficile* species and to differentiate between 027 and non-027 isolates. No ribotype 027 was detected in this region. PCR ribotyping is considered as one of the gold standards to identify 027 and other hyper-virulent strains. Even though ribotypes 017, 001, and 012 are predominant in this region, ribotype 027 is an important genotype being monitored routinely in clinic. In comparison with other available assays, mPCR-CE assay provides another tool to simultaneously detect and differentiate toxigenic *C. difficile* in stool specimens. mPCR-CE assay should also be employed to monitor CDI and *C. difficile* colonization in hospitals and communities in China.

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Compliance with ethics guidelines

Hanjiang Lai, Chen Huang, Jian Cai, Julian Ye, Jun She, Yi Zheng, Liqian Wang, Yelin Wei, Weijia Fang, Xianjun Wang, Yi-Wei Tang, Yun Luo, and Dazhi Jin declare that they have no conflict of interest. This study as a part of the Programs for Major Science and Technology Medicine and Healthcare in Zhejiang (No. WKJ-ZJ-1507) has been approved by the Institutional Review Boards of ZJCDC. This study was presented in part at the 114th American Society for Microbiology Annual Meeting, Boston, MA, 17 to 20 May 2014.

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