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Original Article

Detection of toxin genes from *Clostridium difficile* by using real-time polymerase chain reaction

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Abstract: *Clostridium difficile* infection (CDI) is diagnosed by determining the toxigenic strains of *C. difficile* or its toxins and a clinical examination. We evaluated the performance of four nucleic acid amplification tests (NAATs), which, unlike routine toxigenic culture (TC), enable the determination of toxigenic *C. difficile* directly from the feces. We prospectively examined stool samples from 150 hospitalized adult patients and 141 healthy volunteers. The results from polymerase chain reaction (PCR) were compared to those obtained from TC, direct cytotoxicity test (CTT), ImmunoCard® test, and enzyme-linked immunosorbent assay. Moreover, we evaluated the diagnostic yield of PCR. The sensitivity and specificity of PCR calculated using TC as the gold standard were 100% and 99.2%, respectively. We included 150 patients (men 49.7%; median age, 61 years [range 19-95 years]) during the 2-month period. Most patients were admitted to the medical wards (56%), followed by the surgical (20.7%) and hematology/oncology wards (20.7%), and the intensive care units (2.6%). The patients were categorized according to the laboratory findings as follows: patients with a positive TC (n = 17), of which five had a negative CTT, and patients with a negative TC (n = 133). No differences were observed between patients with positive (n = 12) and negative CTT (n = 5) among the TC-positive group of patients. One patient showed a positive result with PCR. PCR has a high sensitivity for the detection of toxigenic *C. difficile* in CDI; however, this technique cannot be used to differentiate between patients with CDI and asymptomatic carriers.

Keywords: *Clostridium difficile*, toxin genes, PCR, *tcd* A, *tcd* B

Introduction

Clostridium difficile is a gram-positive, spore forming, and anaerobic bacterium. Patients infected with *C. difficile* may be asymptomatic carriers or may develop a life-threatening disease with or without any symptoms of the disease, such as diarrhea. After the discovery of the bacterium in 1935, *C. difficile* was considered to be a component of the fecal flora of newborns and was not thought to be pathogenic [1].

The diagnosis of *C. difficile* infection (CDI) requires the detection of toxigenic *C. difficile* or its toxins and a clinical assessment. Over the past decade, the prevalence and severity of CDI have increased because of the outbreaks of epidemic strains of *C. difficile*, particularly the BI/NAP1/027 strain (polymerase chain reaction [PCR] ribotype 027) and PCR ribotype 078 [2, 3]. Typically, CDI is diagnosed on the basis of the clinical history of patients, including recent antimicrobial usage and occurrence of diarrhea, and the findings from laboratory tests [4]. The direct cytotoxicity test (CTT) is the gold standard in laboratory diagnosis; however, toxigenic culture (TC) has recently been used for the diagnosis of CDI [5].

Nucleic acid amplification tests (NAATs) that detect the toxin genes (usually the toxin B gene, *tcd B*) present on the chromosomes have high sensitivity and specificity, provide rapid results, and can be used for batch and on-demand testing; however, recent guidelines do not recommend these tests for routine use. The guidelines for determining *C. difficile* toxin genes include two-step algorithms that use glutamate dehydrogenase (GDH) assays to screen for *C. difficile* in stool specimens followed by either direct cytotoxin testing or culture to identify

toxin-producing *C. difficile* isolates or GDH algorithms or NAATs [6]. The high molecular weight toxins *tcd A* (308 kDa) and *tcd B* (269 kDa) belong to the family of large clostridial toxins. These toxins possess glucosyltransferase activity, which targets small GTP-binding proteins or guanosine triphosphate (RhoA, Rac, and Cdc42). Glycosylation of these proteins interferes with the signaling pathways in enterocytes and with actin cytoskeleton modifications, induces disruption of tight junctions, and has a cytopathic effect [7]. Highly virulent strains of *C. difficile* have several mutations in the *tcd C* gene. PCR ribotype 027 harbors an 18-bp in-frame deletion and a single nucleotide (nt) deletion at position 117 (Δ117) in the *tcd C*, whereas PCR ribotype 078 (and 066) possesses a 39-bp in-frame deletion and a single nt substitution at position 184 (C184T) in *tcd C* [8, 9]. Toxigenic ribotypes of *C. difficile* may produce one or both of the major exotoxins *tcd A* and *tcd B*. To date, our knowledge about the role of the toxins in CDI is based on results obtained from studies using animal models. Unlike *tcd B*, *tcd A* causes fluid accumulation in various animal models [10]. In addition to *tcd A* and *tcd B*, epidemic strains (including PCR ribotypes 027, 066, and 078) may express a third toxin, namely the binary toxin (actin-ADP-ribosylating toxin *C. difficile* transferase, [CDT]), encoded by *cdt* A and *cdt* B located in the *Cdt* locus [11-13].

Materials and methods

Setting and specimens

The study was performed in a tertiary teaching hospital with approximately 30,000 patients admitted annually. Initial PCR evaluation was performed on 45 culture-positive stool samples. Thereafter, consecutive stool specimens that were submitted to the routine clinical microbiology laboratory for detection of *C. difficile* toxins were collected prospectively from 150 hospitalized adult patients. Only one specimen per patient was included. The infection control unit of our hospital recorded no outbreaks of diarrheal pathogens in the study period. Upon receipt at the laboratory, the routinely used diagnostic ImmunoCard® Toxin A and B test (ICTAB; Meridian Bioscience, Cincinnati, OH, USA) was performed and four aliquots of the stool specimen were stored at -80°C for subsequent testing in batches by using TC, PCR, CTT, and enzyme-linked immunosorbent assay (ELISA).

To establish a correlation between the test results and clinical presentation of patients, we collected data about their signs and symptoms. To assess the presence of asymptomatic carriers, we recruited healthy adult volunteers from the medical students and hospital employees. The volunteers were informed about the purpose of the study and that the results would not be reported. The exclusion criteria for volunteers were the use of antibiotics or complaints of diarrhea. All specimens were thawed only once before testing in a batch. Specimens from all volunteers were tested using TC and PCR, and positive specimens were examined further by using ICTAB, ELISA, and CTT. The study was approved by the ethic committee of Xinxiang City Central Hospital, Henan, China.

Routinely used enzyme immunoassay

The routinely used diagnostic ICTAB test ImmunoCard® (Meridian Bioscience) was performed following the manufacturer's instructions. The results were interpreted independently by two technicians.

TC

C. difficile selective agar with cefoxitin, amphotericin B, and cycloserine (CLO; bioMérieux, Marcy l'Etoile, France) and Columbia blood agar with colistin and nalidixic acid (CAP, Oxoid, Cambridge, UK) were inoculated with 10 µL of the stool specimen. The media were incubated for 5 days under anaerobic conditions at 37°C. Colonies with growth characteristics of *C. difficile* were examined using sequence analysis of the 16S rRNA for identification [14, 15]. *C. difficile* isolates were subcultured in the brain heart infusion (BHI, CM0225, Oxoid) for the determination of toxin production using the cytotoxicity test and PCR detecting genes encoding toxin A and B.

CTT

The CTT was used to determine the presence of toxins in stool samples (direct CTT) and toxin production in *C. difficile* isolates (TC). The supernatant obtained from 1 mg stool sample suspended in 1 mL phosphate-buffered saline (PBS) was filtered through a 0.45-µM filter (Millipore®, Billerica, MA, USA). We incubated Vero cells with 20 µL of the filtrate and 20 µL of

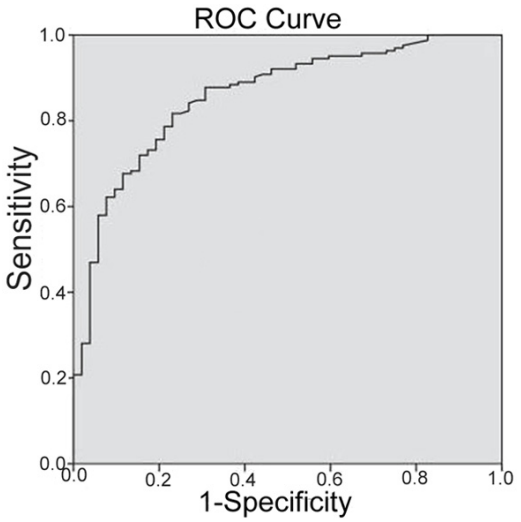

Figure 1. ROC curve analyses of *Clostridium difficile* toxin in patients (n = 150) versus controls (n = 141). AUC = 0.849, Cut-off = 7.09 ng/μl (Kit = 7.20 ng/μl).

the filtrate with 20 μL of anti *C. sordellii* antitoxin (SAT; Uniprom, T5000, Krimpenaan de IJssel, the Netherlands) for 48 h at 37°C. Appropriate controls were included with each microtiter plate used, i.e., 20 μL of toxin (*C. difficile* Bess strain), 20 μL of SAT, and 20 μL toxin with 20 μL SAT. The specimen was positive if 50% of the cells showed a characteristic cytopathic effect (cell rounding), which was neutralized by SAT. The cytotoxicity of the isolates was tested as described above using a 25-μL aliquot of BHI after filtering through a 0.45-μM filter.

PCR

We used 200 μL of the supernatant of a 10% fecal suspension in PBS for DNA isolation using the MagNA Pure LC using the MagNA Pure LC Total Nucleic Acid Isolation K it (Roche Diagnostics, Almere, Netherlands). For PCR analysis of cultured bacteria, a single colony was boiled for 10 min in 50 μL of glycerol broth. We used 5 μL of the prepared DNA for the PCR. Real-time PCR (RT-PCR) assay: a triplex RT-PCR assay was developed to simultaneously detect the *C. difficile tcd A* and *tcd B* toxin genes and the gB polymerase gene of phocine herpesvirus (PhH V-1), which served as the internal control [16]. A positive PCR result did not distinguish between the presence of the toxin A and/or B gene; *tcd A* primers and probes were adapted from the method described by Bélanger et al. [17], i.e., the anti-sense molecular beacon was replaced by a sense TaqMan probe (5'-CTACACTATAgAggAAgAgATTCAAAATCCTCA-3'); *tcd* B and PhHV primers and probes were used according to that described in a previous study [18, 19]. PCR mixes consisted of 25 μL of 2 × LC480 Probes Master (Roche Diagnostics Corporation, Indianapolis, USA), 0.5 μM of each primer, and 0.1 μM of each probe and 5 μL of extracted DNA. RT-PCR was performed on a Roche LC480. We used DNA sequencing to confirm the identity of *C. difficile*; 16S rRNA gene sequences were determined using the primers described by Weisburg et al. [14]. All DNA sequences were determined using an EpiNext High-Sensitivity Bisulfite Seq Kit (Illumina, New York, USA).

Automated NAATs

The Illumigene assay is based on loop-mediated isothermal amplification (LAMP), whereas GeneXpert is a multiplex RT-PCR assay. For the Illumigene assay, external quality control was performed once a day and for GeneXpert for each lot of the kit used a negative control (NC) (w/o stool) and a positive control (PC) (toxigenic *C. difficile* fecal sample). The analysis of inhibited samples was repeated with a reduced fecal load until we obtained a valid result.

Independent DNA extraction for manual NAATs: NucliSENS® easyMAG®

Nucleic acid extraction from the stool sample was performed using the NucliSENS® easyMAG® platform (bioMérieux), according to the manufacturer's protocol optimized for stool samples ("Extraction Protocol for the NucliSENS easyMAG BTL039444 rel. 1.0 for stool samples" in combination with "Specific B" protocol). Stool samples were transferred to NucliSENS® Lysis Buffer (bioMérieux) [1:2 (wt/vol)], vortexed and homogenized for 1 min at 7,000 rpm using the MagNA Lyser Instrument (Roche Applied Science, Penzberg, Germany). Fecal suspensions were centrifuged for 2 min at 16,000 *g* and stored at -20°C until batch testing. The automated extraction was performed in batches of 24 samples. We thawed and re-spun 100 μL of the supernatant and incubated it in 2 mL of NucliSENS® Lysis Buffer for 10 min at room temperature; the PhHV was used as the internal extraction and amplification control (IC) in the in-house algo-

Table 1. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) using toxigenic culture (TC) as the gold standard

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Polymerase chain reaction (PCR)	100	98.12	94	98
Direct cytotoxicity test (CTT)	61	100%	100%	93.1
ImmunoCard® Toxin A and B (ICTAB)	49	91	81	84.3
Enzyme-linked immunosorbent assay (ELISA)	51	83	65	90.1

Table 2. Characteristics and clinical data of patients with positive and negative toxigenic culture (TC)

	TC-positive (n = 17)	TC-negative (n = 133)[a]
Fever > 38.5°C	5/16 (31%)	50/127 (39%)
Diarrhea		
> 48 h	9/17 (53%)	64/133 (49%)
> 24 h	2/17 (11%)	64/133 (49%)
No	6/17 (36%)	46/133 (34%)
Abdominal pain	7/16 (44%)	33/127 (26%)
Gastric tube feeding	3/17 (18%)	27/133 (20%)
Chemotherapy	2/17 (12%)	21/133 (16%)
Prior CDI	2/17 (12%)	21/133 (16%)
Proton pump inhibitor	8/17 (47%)	50/133 (38%)
Immunocompromised	7/17 (42%)	62/133 (47%)
WBC		
> 15 × 10/l	6/17 (35%)[b]	14/133 (10%)[b]
< 15 × 109/l	9/17 (53%)	9/17 (53%)
Unknown	2/17 (12%)	9/133 (7%)
CRP		
< 5 mg/l	3/17 (18%)	11/133 (8%)
> 15 mg/l	> 15 mg/l	83/133 (62%)
5-15 mg/l	1/17 (6%)	17/133 (13%)
Unknown	3/17 (18%)	22/133 (17%)

CDI: *Clostridium difficile* infection; ICU: intensive care unit; WBC: white blood cell count; CRP: C-reactive protein. [a]Including one patient with a positive PCR only; [b]$P < 0.05$.

rithm. We loaded 2.1 mL of the sample together with 140 µL of magnetic silica onto the platform. The output eluates (110 µL) were stored at 5°C until the weekly manual RT-PCR analysis.

Clinical evaluation

Demographic data and laboratory results were collected from the electronic patient files. We collected the medical history and current signs and symptoms of the patients by interviewing the attending physician and reviewing the medical charts using a standardized questionnaire. Data collected included reason for and duration of admission; characteristics of the current episode of diarrhea (consistency, frequency, duration, and abdominal pain); levels of C reactive protein (CRP) and white blood cell count (WBC); use of antibiotics in the previous 3 months; and the presence of other risk factors for *C. difficile* infection (previous hospitalization, use of immunosuppressive medication, proton pump inhibitors, gastric tube feeding, chemotherapy, and abdominal surgery).

We retrospectively evaluated the clinical course in patients who were negative by ICTAB but were positive by PCR to investigate the clinical significance of these positive results.

Statistical analysis

For the statistical comparison of routine toxigenic culture-negative samples, a regression method for paired samples was used [18]. The outputs were relative positive fractions with 95% confidence intervals (CIs). For tabulated data with low or zero counts, Fisher's exact test and McNemar's test were used. A P value of 0.05 was considered significant. The statistical calculations were performed in the open access software R. The Fisher test command in R allows for tables larger than 2 × 2.

Ethical approval

The study protocol was approved by the ethics committee of Shandong Provincial Qianfoshan Hospital, Jinan, China. Written informed consent was obtained from all patients before the start of the study. The study was conducted according to the International Conference on Harmonization (ICH) Good Clinical Practices (GCP) and in compliance with the Declaration of Helsinki and subsequent amendments.

Results

All of the 45 culture-positive stool samples from the initial evaluation were positive on PCR

Table 3. Primers and probes for the in-house real-time (RT) polymerase chain reaction (PCR)

Gene target	Sequence name	Sequence (5'-3')[a,b]	Final concentration (nM)	Reference
Toxin reaction				
tcd A	*tcd* A-F	AATTTAGCTGCTGCAGCATCTGACATAGT	300	(Hoegh et al. 2011)
	tcd A-R	TTCCCAACGGTCTAGTCCAATAG	300	(Hoegh et al. 2011)
	tcd A-P	VIC-TGTTGATATGCTTCCAGGTAT-MGB	100	(Hoegh et al. 2011)
tcd B	*tcd* B-F	ATAATGGTAGATTTATGATGGAACTAGGAA	300	(Hoegh et al. 2011)
	tcd B-R	TCTTGATAAGCTGCCGCATATG	300	(Hoegh et al. 2011)
	tcd B-P	FAM-AGAGTTGGTTTCTTCCCAG-MGB	100	(Hoegh et al. 2011)
cdt A	*cdt* A-F	ATGTAAATGATTATATGCGTGGAGGAT	100	(Hoegh et al. 2011)
	cdt A-R	GGTTCACGTTTTTAATGCATTTTCA	300	(Hoegh et al. 2011)
	cdt A-P	NED-TCAAATGGTCCAGTAAAT-MGB	100	(Hoegh et al. 2011)
PhHV	PhHV-F	GGGCGAATCACAGATTGAATC	80	Niesters HG (2001)
	PhHV-R	GCGGTTCCAAACGTACCAA	100	Niesters HG (2001)
	PhHV-P	CY5-TTTTTATGTGTCCGCCACCATCTGGATC-BBQ	100	Niesters HG (2001)
tcd C genotyping				
tcd C	CD-*tcd* C-F	GCACAAAGGRTATTGCTCTACTGG	300	(de Boer et al. 2010)
	CD-*tcd* C-R1	AGCTGGTGAGGATATATTGCCAA	300	(de Boer et al. 2010)
	CD-*tcd* C-R2	CAAGATGGTGAGGATATATTGCCA	300	(de Boer et al. 2010)
	tcd C-wtAP	FAM-CACGCCTAAAATAA-MGB	100	Present study
	tcd C-wtGP	FAM-ACGCCCAAAATA-MGB	100	Present study
	tcd C-Δ117	VIC-AACACACCAAAATAA-MGB	100	Present study
	tcd C-A117T	NED-ACACACCAAAAATAA-MGB	100	Present study

[a]MGB minor groove binder; BBQ Blackberry quencher. [b]R = A or G.

testing. We included 150 patients (49.7% men; median age, 61 years [range 19-95 years]) during the 2-month period. Most patients were admitted to the medical wards (56%), followed by the surgical (20.7%) and hematology/oncology wards (20.7%), and the intensive care units (2.6%). Routine examination of the anaerobic culture yielded 42 *C. difficile*-positive samples from 39 patients. Genotypic PCR analysis of *C. difficile* isolated from three samples showed that they were non-toxigenic; similarly, the results obtained using the four NAATs showed that the samples were negative. One isolate could not be recovered and was excluded from analysis. Therefore, the prevalence of toxigenic *C. difficile* estimated by routine culture was 12.7% (38 out of 299). ROC case-control analysis showed a cut-off value of 7.09 ng/μl (Kit = 7.20 ng/μl) with an AUC of 0.849 (**Figure 1**). The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the PCR, ICTAB, ELISA, and CTT obtained using TC as the gold standard are shown in **Table 1**. On the basis of the laboratory findings, the patients were categorized as those with a positive TC (n = 17), of which five had a negative CTT and a negative TC (n = 133). We compared characteristics and clinical data of the patients in these categories (**Table 2**). Patients with a positive TC ($P < 0.05$) showed a high frequency of previous antibiotic use and a high occurrence of elevated WBC. No differences were observed between patients with positive (n = 12) and negative CTT (n = 5) among the TC-positive patients; however, the number of patients was low. Only one patient showed a positive result with PCR alone. The primers and probes for the in-house RT-PCR are listed in **Table 3**.

The total inhibition rate of PCR (18.4%) was significantly ($P < 0.001$) higher than that achieved with other assays. The specimens that initially yielded an invalid result were reexamined using a diluted template or a sample with reduced fecal load until a valid result was recorded, and this result was used to characterize the performance of the method. The specificity ranged from 92.0% to 98.1%, with no significant difference between the tests. PCR showed the high-

Table 4. Discrepant results between routine toxigenic culture-positive samples and NAATs results

Sample ID	Routine culture	PCR ribotype	Toxinotype	*ICTAB*	*Direct cytotoxicity test*	*Polymerase chain reaction*	Independent DNA extraction for manual NAATs: NucliSENS® easyMAG®
F-2549	*C. difficile*	027	III	Positive	027	Negative	027
F-2646	*C. difficile*	027	III	Positive	027	Negative	027
F-6368	*C. difficile*	027	III	Positive	027	Negative	027
F-6544	*C. difficile*	027	III	Positive	027	Negative	027
F-2243	*C. difficile*	SLA0008	0	Positive	Non-027	Negative	Non-027
F-4743	*C. difficile*	SLA0005	0	Positive	Non-027	Negative	Non-027
F-7531	*C. difficile*	095	0	Positive	Non-027	Negative	Non-027
F-4324	*C. difficile*	SLA0010	0	Negative	Non-027	Negative	Neg
F-6452	*C. difficile*	014/020/077	0	Negative	Non-027	Negative	Non-027

[a]Toxigenic *C. difficile*. [b]Toxigenic *C. difficile*, 027 potential 027 PCR ribotype; Non-027 potential non-027 PCR ribotype.

est specificity. However, this assay had a significantly ($P < 0.001$) lower sensitivity (76.3%). ICTAB, CTT, and PCR had sensitivities of 94.7%, 97.4%, and 100%, respectively. The results from false-negative samples (routine toxigenic culture-positive samples that failed to be detected by an NAAT) are shown in **Table 4**.

Discussion

Between 2003 and 2008, hospitals across 40 states in the US and in the Canadian provinces reported the cases of patients with CDI caused the 027/BI/NAP1 strain. A multicenter clinical trial performed in 2005 on the toxin-binding polymer tolevamer showed that the 027/BI/NAP1 strain accounted for 36% of all strains collected in the trial. The results of two clinical trials on fidaxomicin performed between 2006 and 2009 showed that the incidence of the 027/BI/NAP1 strain among the American and Canadian population was 38.1% and 45.9%, respectively [20, 21].

To date, the diagnosis and prevention of CDI remains an important concern for public health because of the recent emergence of infecti-ous strains of *C. difficile*. The spread of *C. difficile* can be avoided by applying strict measures. Prevention can be achieved by contact isolation. The patient should be nursed preferably in a single bedroom with dedicated equipment, gloves, and gowns. Isolation is key in controlling the outbreak of CDI. One of the most important measures for avoiding the spread of CDI is cleaning and disinfecting the environment that may be contaminated by patients with CDI. Hypochlorite-based disinfectants are recommended for routine use. Hypochlorite used at a concentration of 1000 parts per million significantly decreases the incidence of CDI. A recent study showed that hydrogen peroxide vapor is effective for eradication of *C. difficile* in the environment [22].

The pathogenesis of CDI has significantly changed over the last decade since the spread of new epidemic strains, and the incidence of CDI has increased worldwide.

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Disclosure of conflict of interest

None.

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