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Investigation of toxin gene diversity and antimicrobial resistance of *Clostridium difficile* strains

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Abstract. The incidence of *Clostridium difficile* infection (CDI) has been previously reported in a number of studies. However, data collected from the Chinese population is limited. In the present study, the diversity of the toxin genes, *tcdA* and *tcdB*, of 57 *Clostridium difficile* (*C. difficile*) isolates from a Chinese population were investigated by polymerase chain reaction (PCR) (38 A^+B^+, 14 A^-B^+ and 5 A^-B^-). Quantitative PCR was used to check the expression of these two genes and it was found that the genes were not expressed by all the strains. The absence of *tcdA* or *tcdB* expression in certain strains could be due to the lower expression of *tcdD* and the higher expression of *tcdC*, which are positive and negative regulators for these two toxin genes, respectively. In addition, the antimicrobial susceptibilities of 57 isolates were investigated. Therefore, these data would aid in the future prevention of CDI outbreaks and improve the understanding of the infection.

Introduction

Clostridium difficile (*C. difficile*) has been associated with a wide range of diseases, including toxic megacolon, nosocomial diarrhea and pseudomembranous colitis (1,2). Toxigenic and epidemic *C. difficile* is a well-established health threat in the nosocomial environment. Several studies have shown that *C. difficile* causes community-acquired infections and it has been isolated from various human, animal, food and environmental sources, often with similar genetic profiles (3,4). The pathogenicity of *C. difficile* is associated with its ability to produce two toxins: Toxin A, an enterotoxin; and toxin B, a potent cytotoxin (5), which are responsible for the cellular damage linked to diseases. The *tcdA* and *tcdB* genes that encode toxins A and B, respectively, are located in the pathogenicity locus (*PaLoc*), along with the positive and negative regulator genes, *tcdD* and *tcdC*, respectively (6). Several polymerase chain reaction (PCR) methods have been developed to detect the *tcdA* and *tcdB* genes and there have been certain studies of the toxin gene diversity, molecular epidemiology and antimicrobial resistance of *C. difficile* isolated from hospitals. However, thus far, limited data are available on the toxin gene diversity and antimicrobial susceptibilities of the bacterium isolated from *C. difficile* infection (CDI) patients in China. In the present study, *C. difficile* isolates were analyzed from patients in the Central Hospital of Taizhou City (Taizhou, China) for the presence of the *tcdD*, *tcdC*,*cdtA* and *cdtB* genes and the expression patterns were examined via quantitative PCR (qPCR). Additionally, the susceptibility of the *C. difficile* profiles to 12 antimicrobial agents, including nemonoxacin and tigecycline, were investigated.

Materials and methods

Identification of C. difficile isolates. The faecal samples were collected from various departments of the Taizhou Central Hospital. Isolation of *C. difficile* was performed on selective Columbia agar supplemented (bioMerieux Co., Ltd., Shanghai, China) with cycloserine-cefoxitin and amphotericin B (Bayer Co., Ltd., Shanghai, China) as described previously (7). Briefly, the plates were incubated in an anaerobic chamber at 37˚C for 72 h. The *C. difficile* isolates were identified by colony morphology, Gram staining, odor and green-yellow fluorescence under UV light (365 nm).

PCR assays. All the PCR reactions were performed with a positive and negative control using the primers (Table I) described by previous studies (8,9). PCR was conducted with 2.5 pl cDNA and 15 pmol of each primer pair in a total volume of 50 p1 with 1 unit of Taq DNA polymerase (Takara Bio, Inc., Shiga, Japan) in a standard reaction mixture. Amplification was achieved by denaturing at 95˚C (1 min), primer annealing at 52˚C (1 min) and extension at 72˚C (1 min), which were repeated for 30 cycles.

RNA extraction, cDNA synthesis and qPCR assays. cDNA synthesis was performed as described previously by Frias-Lopez *et al* (9). RNA was extracted from cultured bacterial cells using AllPrep DNA/RNA mini kit (Qiagen,

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Hilden, Germany) following the manufacturer's instructions and 2-3 μg RNA was expected to be obtained. To eliminate the potential contamination by DNA, the TURBO DNA-free™ kit was utilized (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Subsequently, RNA was reverse transcribed into first strand cDNA using the SuperScript III First-Stand Synthesis SuperMix kit (Invitrogen Life Technologies, Carlsbad, CA, USA) and random hexamer priming. qPCR was performed using the KAPA SYBR FAST qPCR kit (Kapa Biosystems, Woburn, MA, USA) following the manufacturer's instructions: Denaturing at 95˚C (1 min), primer annealing at 52˚C (1 min) and extension at 72˚C (1 min), repeated for 30 cycles. The increase in fluorescence was measured in real-time during the extension step. The primers used are listed in (Table I). The ΔCt values for each sample between the toxin genes and 16S rRNA were calculated and are listed in Table II. The relative expression levels of *tcdC* and *tcdD* were calculated based on the ΔCt values between the two genes and 16S rRNA.

Antimicrobial susceptibility testing. The antimicrobial susceptibility tests were performed with 57 *C. difficile* isolates as described previously (10). Briefly, an inoculum of 10^5 CFU bacteria was applied to each plate with a glass replicator on supplemented Brucella blood agar (11). The plates were incubated in an anaerobic chamber for 48 h at 37˚C. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of each antimicrobial agent that inhibited the growth of the tested isolate. The antimicrobial agents (Sigma-Aldrich, St. Louis, MO, USA) used are listed in Table III.

Table I. Primers used in the present study.

Primers	Oligonucleotide sequence (5'→3')
tcdA-PCR-F	CCCAATAGAAGATTCAATATTAAGCTT
tcdA-PCR-R	GGAAGAAAAGAACT TCTGGCTCACTCAGGT
tcdB-PCR-F	GGTGGAGCTGCTTCATTGGAGAG
tcdB-PCR-R	GTGTAACCTACTTTCATAACACCA
tcdA-qPCR-F	TCTACCACTGAAGCATTAC
tcdA-qPCR-R	TAGGTACTGTAGGTTTATTG
tcdB-qPCR-F	ATATCAGAGACTGATGAG
tcdB-qPCR-R	TAGCATATTCAGAGAATATTG
tcdC-qPCR-F	TCTCTACAGCTATCCCTGGT
tcdC-qPCR-R	AAAAATGAGGGTAACGAATTT
tcdD-qPCR-F	CTCAGTAGATGATTTGCAAGAA
tcdD-qPCR-R	TTTTAAATGCTCTATTTTTAGCC

qPCR, quantitative polymerase chain reaction; F, forward; R, reverse.

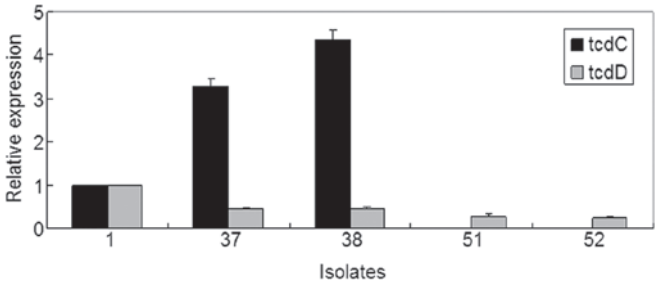

Figure 1. Quantitative polymerase chain reaction detection of the *tcdC* and *tcdD* genes in the *Clostridium difficile* isolates.

Results

Analysis of the toxin genes, tcdA and tcdB. *C. difficile* were isolated from various departments of the hospital: 20 from the Department of Neurosurgery, 7 from the Intensive Care Unit, 10 from the Department of Infectious Diseases, 10 from the Department of Hematology and 10 from the Department of Radiation Oncology (Table II). The PCR assay was used to differentiate toxin A-negative and B-positive (toxin A^-, toxin B^+) strains from the toxin-positive (toxin A^+, toxin B^+) strains and the toxin-negative (toxin A^-, toxin B^-) strains. The primers used are listed in Table I. As shown in Table II, 38 and 14 isolates of the A^+B^+ and A^-B^+ strains were identified, respectively, which were the toxigenic strains. The recovery rates of the toxigenic strains were 85-100% according to the hospital studied. By contrast, 5 isolates were A^-B^-. To investigate the expression levels of *tcdA* and *tcdB* genes, qPCR was performed. The Ct values were summarized in Table II. Of the total 38 *tcdA* PCR-positive isolates, 36 could be detected for the expression of this gene and the expression level varied slightly between the 36 transcripts. Of the total 52 *tcdB* PCR-positive isolates, the expression of this gene could be identified in 50 qPCR transcripts and they showed slight variations in the expression level as well. No transcription could be detected in the A^-B^- isolates.

Detection of the tcdC and tcdD genes. Based on these results, the transcription of *tcdA* could not be detected in isolates 37 and 38, and the transcription of *tcdB* could not be detected in isolates 51 and 52. As mentioned above, TcdD and TcdC have been indicated as the positive and negative regulators of toxin A and B expression, respectively (6). In order to investigate why the *tcdA* or *tcdB* genes are not expressed in isolates 37, 38, 51 and 52, qPCR was performed to detect the expression of *tcdC* and *tcdD*. Isolate 1, which showed a high expression of the *tcdA* and *tcdB* genes, was used as a positive control. The results (Fig. 1) showed that the mRNA level of *tcdD* was lower in isolates 37 and 38 compared to isolate 1, and by contrast, the *tcdC* mRNA level was relatively higher. Furthermore, the mRNA level of *tcdD* was notably lower in isolates 51 and 52, whereas no transcription of *tcdC* was detected in these two isolates.

Antimicrobial susceptibilities. The MIC ranges, MIC50s, MIC90s and the percentages of the susceptibility of 57 *C. difficile* isolates to 12 antimicrobial agents are summarized in Table III. All the isolates were susceptible to vancomycin (MIC, ≤2 μg/ml). Susceptibility to piperacillin, ampicillin-sulbactam, imipenem, meropenem, metronidazole and tigecycline was shown in >80% isolates. The resistance rates to cefotetan, moxifloxacin and ertapenem were >70%. However, no isolates

Table II. PCR and qPCR detection of the *tcdA* and *tcdB* genes.

				qPCR					
		PCR		*tcdA*			*tcdB*		
Isolates	Unit	*tcdA*	*tcdB*	ΔCt1	ΔCt2	ΔCt3	ΔCt1	ΔCt2	ΔCt3
1	DN	+	+	2.90	2.75	2.66	5.76	5.33	5.53
2	DN	+	+	2.11	2.23	2.83	4.32	4.36	4.57
3	DN	+	+	1.56	1.58	1.63	5.23	5.55	5.34
4	DN	+	+	2.52	2.67	2.54	2.98	2.75	2.54
5	DN	+	+	2.70	2.65	2.63	4.87	4.54	4.34
6	DN	+	+	2.55	2.44	2.48	3.21	3.45	3.65
7	DN	+	+	2.33	2.35	2.37	3.35	3.45	3.37
8	DN	+	+	3.64	3.70	3.72	3.34	3.75	3.44
9	DN	+	+	2.31	2.11	2.34	5.43	5.46	5.67
10	DN	+	+	1.06	1.05	0.91	4.83	4.92	4.44
11	DN	+	+	1.01	1.09	1.13	5.43	5.55	5.57
12	ICU	+	+	0.05	0.03	0.02	6.71	6.32	6.43
13	ICU	+	+	2.13	2.14	2.23	5.31	5.21	5.55
14	DN	+	+	2.37	2.39	2.30	5.73	5.77	5.78
15	DN	+	+	3.68	3.72	3.71	4.32	4.33	4.37
16	DN	+	+	2.97	2.95	2.92	3.21	3.22	3.34
17	DN	+	+	3.21	3.24	3.26	2.34	2.37	2.39
18	DN	+	+	1.09	1.11	1.13	1.29	1.27	1.25
19	DN	+	+	1.21	1.22	1.19	2.32	2.35	2.42
20	DN	+	+	1.24	1.29	1.27	2.55	2.57	2.60
21	DD	+	+	0.36	0.34	0.39	3.21	2.98	3.02
22	DD	+	+	1.02	1.03	1.05	4.56	4.57	4.58
23	DD	+	+	2.01	2.07	1.98	2.22	2.25	2.29
24	DD	+	+	3.03	3.05	3.07	4.59	4.54	4.56
25	DD	+	+	4.51	4.55	4.52	3.37	3.41	3.21
26	DD	+	+	2.72	2.71	2.69	2.21	2.29	2.31
27	DD	+	+	1.67	1.72	1.69	3.98	3.96	3.92
28	DD	+	+	1.79	1.78	1.78	6.53	6.52	6.32
29	DD	+	+	0.77	0.78	0.80	5.90	5.78	5.32
30	DD	+	+	0.34	0.35	0.36	4.90	4.93	4.53
31	DH	+	+	0.56	0.57	0.59	3.98	3.77	3.63
32	DH	+	+	2.31	2.34	2.35	3.29	3.27	3.25
33	DH	+	+	3.41	3.42	3.42	4.53	4.52	4.50
34	DH	+	+	2.79	2.78	2.77	2.98	2.97	2.93
35	DH	+	+	1.56	1.58	1.54	1.95	1.92	1.93
36	DH	+	+	1.96	1.94	1.93	4.78	4.79	4.70
37	DH	+	+	NA	NA	NA	4.56	4.67	4.98
38	DH	+	+	NA	NA	NA	3.32	3.45	3.56
39	DPE	-	+	NA	NA	NA	5.97	5.67	5.88
40	DPE	-	+	NA	NA	NA	2.60	2.70	2.66
41	ICU	-	+	NA	NA	NA	3.01	3.04	2.34
42	ICU	-	+	NA	NA	NA	3.77	3.58	3.60
43	ICU	-	+	NA	NA	NA	3.62	3.64	3.65
44	DH	-	+	NA	NA	NA	4.21	4.22	4.23
45	DH	-	+	NA	NA	NA	5.32	5.33	5.34
46	DRO	-	+	NA	NA	NA	5.29	5.29	5.27

qPCR, quantitative polymerase chain reaction; DN, Department of Neurosurgery; ICU, Intensive Care Unit; DD, Department of Infectious Diseases; DH, Department of Hematology; NA, not applicable; DPE, Department of Physical Examination; DRO, Department of Radiation Oncology.

Table II. Continued.

				qPCR					
		PCR		*tcdA*			*tcdB*		
Isolates	Unit	*tcdA*	*tcdB*	ΔCt1	ΔCt2	ΔCt3	ΔCt1	ΔCt2	ΔCt3
47	DRO	-	+	NA	NA	NA	3.55	3.57	3.59
48	DRO	-	+	NA	NA	NA	3.11	3.12	3.17
49	DRO	-	+	NA	NA	NA	4.19	4.17	4.16
50	DRO	-	+	NA	NA	NA	2.48	2.47	2.39
51	DRO	-	+	NA	NA	NA	NA	NA	NA
52	DRO	-	+	NA	NA	NA	NA	NA	NA
53	ICU	-	-	NA	NA	NA	NA	NA	NA
54	ICU	-	-	NA	NA	NA	NA	NA	NA
55	DRO	-	-	NA	NA	NA	NA	NA	NA
56	DRO	-	-	NA	NA	NA	NA	NA	NA
57	DRO	-	-	NA	NA	NA	NA	NA	NA

qPCR, quantitative polymerase chain reaction; DN, Department of Neurosurgery; ICU, Intensive Care Unit; DD, Department of Infectious Diseases; DH, Department of Hematology; NA, not applicable; DPE, Department of Physical Examination; DRO, Department of Radiation Oncology.

Table III. Minimal inhibitory concentrations (MICs) of 12 antimicrobial agents for the 57 *Clostridium difficile* isolates.

	MIC (mg/l)			
Antimicrobial agent	MIC50	MIC90	Range	Resistant, %
Vancomycin	0.40	1.50	0.28-2.00	0.00
Piperacillin	4.20	26.00	0.92-33.00	7.02
Ampicillin-sulbactam	1.65	6	0.24-10.00	8.77
Imipenem	10.00	18.20	1.9-32.00	10.00
Meropenem	3.20	10.20	0.95-15.00	8.77
Metronidazole	0.80	2.56	0.125-5.50	17.54
Tigecycline	0.08	0.10	0.03-0.36	17.54
Cefotetan	35.00	168.00	1.8-185.00	21.05
Moxifloxacin	2.80	37.00	0.9-175.00	63.15
Ertapenem	4.00	39.00	0.03-67.00	87.7
Cefoxitin	101.00	145.00	45-156.00	100.00
Clindamycin	95.00	267.00	4-333.00	100.00

were susceptible to cefoxitin or clindamycin. Tigecycline demonstrated the lowest MIC50 (0.08 mg/l) and inhibited all the strains at 0.36 mg/l, whereas cefoxitin showed the highest MIC50 (101 mg/l). Tigecycline was also the most active with regards to MIC90 (0.1 mg/l), whereas clindamycin had the highest MIC90 (267 mg/l).

Discussion

The toxin gene diversity has been investigated in numerous studies by PCR (10). For example, the multiplex PCR method by Persson *et al* (14) allowed the simultaneous identification of the *tcdA*, *tcdB*, *cdtA* and *cdtB* toxin genes. In addition, a multiplex qPCR method for the detection of toxigenic *C. difficile* from stools and the presumptive identification of the NAP-1 strain was developed by Jayaratne *et al* (15). In the present study, PCR and qPCR were carried out to identify the *tcdA* and *tcdB* toxin genes in 57 isolated samples from the Central Hospital of Taizhou City, and the study was a systematic survey of the types of the *C. difficile* toxin genes in China. The results showed that of the 57 isolates, 38 (66.67%) were A^+B^+, which plays a major role in CDI. A total of 14 (24.56%) isolates were A^-B^+ strains, which have been reported to be significantly increased during recent years (16). In studies from various global locations, different proportions of the A^-B^+ strains have been reported (17,18). In the present study, based on the PCR results, the A^-B^- strain accounted for only 5 (8.77%) of the isolates. However, according to the qPCR results, not all the

A^+ or B^+ isolates showed detectable expression of these genes. This can be explained by the inhibition of *tcdA* or *tcdB* transcription by regulators in certain strains. By contrast, it has also been reported that there are certain activators, including σ factors and the positive regulator TcdD, which are necessary for the expression of TcdA and TcdB (19). Therefore, the absence of these types of activators may be another reason why these two genes are not expressed. However, this requires further investigation.

Certain studies (20) have focused on the regulation of the *tcdA* and *tcdB* toxin genes. Earlier studies (21) indicated that TcdC has a negative influence on the transcription of the other genes in PaLoc, consisting of the *tcdA-E* genes, and TcdD has a positive regulatory function on the transcription of the *tcdD*, *tcdB*, *tcdE* and *tcdA* genes. In the present study, the mRNA levels of the *tcdC* and *tcdD* genes were detected in isolates 1, 37, 38, 51 and 52 to identify why *tcdA* or *tcdB* are not transcribed. Consistent with previous studies (22), the mRNA level of *tcdC* was significantly higher in isolates 37 and 38, in which the toxin *tcdA* was not detectable, indicating that the transcription of *tcdA* could be inhibited by TcdC. Notably, no transcription of *tcdC* could be detected in isolates 51 and 52, possibly due to the absence of this gene in the strains. Spigaglia *et al* (23) have previously reported the deletion of *tcdC* in *C. difficile* clinical isolates. By contrast, another reason why *tcdA* is not expressed in isolates 37, 38, 51 and 52 could be due to the low expression of *tcdD*.

The susceptibility of the 57 *C. difficile* isolates to 12 antimicrobial agents was also investigated. All the isolates of *C. difficile* showed susceptibility to vancomycin, which is consistent with the fact that it is an effective agent against *C. difficile* infection (24). In addition, the *C. difficile* isolates in the present study were universally susceptible to piperacillin, ampicillin-sulbactam, imipenem and meropenem. Based on the study by Settle *et al* (25), it has been proved that piperacillin, regarding its broad-spectrum activity particularly against anaerobes, was relatively more likely to induce *C. difficile* colonization or diarrhea. Thus, it is not widely used in hospitals. Notably, 90% of the isolates in the present study were susceptible to imipenem, which varies from the results of previous studies (12,26). In an investigation of the inhibitory activity of antimicrobial agents against clinical isolates of *C. difficile* by Cheng *et al* (26), found resistance to imipenem in the majority of tested strains. Ampicillin-sulbactam has been reported to be active against *C. difficile* in numerous studies (27,28). Similar to the results in the present study, Lin *et al* (28) and Hecht *et al* (29) also demonstrated that meropenem had low MIC90 values (4 and 2 μg/ml, respectively). Tigecycline had the lowest MIC90 value for *C. difficile* isolates and was followed by vancomycin and metronidazole (all, >3 mg/l), which is similar to previous studies (30,31). Tigecycline has been proved to not induce proliferation or cytotoxin production by epidemic *C. difficile* strains, and patients with severe refractory CDI were successfully treated with tigecycline (32,33). Clindamycin showed the highest MIC90 of all the antimicrobial agents tested, consistent with the study by Lin *et al* (28) and as previously reported by Critchley (34), the use of clindamycin was independently associated with the infection of *C. difficile*.

In conclusion, the present study identified the presence of the *tcdA* and *tcdB* toxin genes in the isolates of 57 *C. difficile* isolates from Chinese patients, using PCR and qPCR. Similar to previous studies, the A^+B^+ was the dominant ribotype. Certain isolates were shown to be lacking *tcdA* or the *tcdB* gene expression, possibly due to the absent or higher expression of *tcdC* and lower expression of *tcdD*. The susceptibility of the genes to 12 agents was also investigated. The isolates showed similar susceptibility to specific agents, as reported previously, such as ampicillin-sulbactam. However, certain agents appeared to be more active against the isolates in the present study compared to in previous studies, such as imipenem. These data provide novel information on the characteristics of *C. difficile* isolated from Chinese patients and would aid in the future prevention of outbreaks.

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