

Comparison of polymerase chain reaction ribotyping, toxinotyping and nutritional aspects of toxin production of *Clostridium difficile* strains

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Abstract. *Clostridium difficile* (*C. difficile*) is the leading cause of infectious diarrhea in hospitals worldwide. Enterotoxin A (TcdA) and cytotoxin B (TcdB), have been identified as the main virulence factors of *C. difficile*. In China, data on polymerase chain reaction (PCR) ribotypes and abilities of hospital-derived *C. difficile* isolates to produce TcdA and TcdB are sparse. In this study, we identified 40 *C. difficile* isolates from the Taizhou hospital and investigated their PCR ribotypes based on the 16S-23S rRNA gene intergenic spacer region. The ability of different ribotypes to produce TcdA and TcdB was determined by immunochromatography and cytotoxicity assays, respectively. The effects of the nutritional status on the production of these toxins were also investigated.

Introduction

Clostridium difficile (*C. difficile*) is one of the causes of antibiotic-associated diarrhea and the leading cause of infectious diarrhea, known as *C. difficile* infection (CDI), in hospitals worldwide (1). Different *C. difficile* strains have been reported in different countries (2,3). Thus, polymerase chain reaction (PCR) ribotyping was developed to determine the relatedness of different strains associated with infection. Stubbs *et al* (4) constructed a library consisting of 116 different *C. difficile* PCR ribotypes of based on PCR targeting of the 16S-23S rRNA gene intergenic spacer region; this library has been used for typing isolates since 1999.

Two toxins, enterotoxin A (TcdA) and cytotoxin B (TcdB), have been identified as the main virulence factors of *C. difficile* (5,6). *C. difficile* strains with different toxins

may be isolated from CDI cases worldwide [both TcdA and TcdB (A+B⁺), only TcdB (A+B⁺) or neither TcdA nor TcdB (A-B)] (7-9). PCR-based toxinotyping may be used to determine the relatedness of different strains. In addition, TcdA and TcdB may be detected by immunochromatography and cytotoxicity assays, respectively (10). The nutritional status of the environment significantly contributes to the ability of this bacterium to grow and produce toxins at a particular time (11,12). Tryptic nitrate broth (TNB) and complete defined medium have been used to quantify TcdA and TcdB under distinct growth conditions (12).

Numerous reports have focused on the ribotypes and toxin profiles of *C. difficile*. However, data on CDI in China are currently sparse. In this study, we isolated 40 *C. difficile* strains from hospitalised patients and identified the ribotypes based on the 16S-23S rRNA gene intergenic spacer region. In addition, the activity of TcdA and TcdB was detected via immunochromatography and cytotoxicity assays, respectively. We also determined the effects of different media on toxin production.

Materials and methods

Ethics statement. The study protocol was approved by the Biomedical Ethics Review Committee of the Taizhou Central Hospital, China. Written informed consent was obtained from all the participating adult patients and from the parents or legal guardians of participating infants.

Samples. Between May, 2012 and December, 2013, a total of 325 stool samples were collected from patients with diarrhea in the Taizhou Central Hospital, China. All the stool samples were prepared for *C. difficile* testing.

Identification of *C. difficile* isolates. Isolation of *C. difficile* was performed on selective Columbia agar (Biomerieux, Shanghai, China) supplemented with cycloserine-cefoxitin and amphotericin B (Bayer, Shanghai, China), as previously described (13). Briefly, *C. difficile* was incubated in an anaerobic chamber: YQX-II (LNB Instrument Company, Qingdao, China) at 37°C for 72 h and the isolates were then

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identified based on colony morphology, Gram staining, odor and green-yellow fluorescence under UV light (365 nm).

PCR ribotyping. All the isolates were typed using the method previously described by Stubbs *et al.* (4). PCR ribotyping was performed by comparing the patterns of the PCR products from the 16S-23S rRNA intergenic spacer region. PCR cycler and electrophoresis system purchased from Thermo Fisher (Waltham, MA, USA) and Bio-Rad (Hercules, CA, USA), respectively. An isolate was considered as a new ribotype if its pattern was different by at least one band from patterns previously described in the library.

Toxin detection. *C. difficile* was grown in brain-heart infusion for 48 h and then centrifuged at 4,000 x g for 10 min to collect the supernatant. TcdA was detected by an immunochromatography assay (Cepheid, Tianjin, China) using anti-toxin A antibodies (14) and TcdB was detected by a cytotoxicity assay on the McCoy cell line according to Pituch *et al.* (10). The cytopathic effect was observed under a microscope following incubation of culture filtrate with McCoy cells. The result was considered to be positive when the cytopathic effect could be neutralized by the polyclonal antiserum to *C. difficile*.

Growth in TNB and complete defined medium and toxin quantification. TNB, which consisted of 20% (w/v) Bacto Tryptose, 0.1% (w/v) glucose, 0.2% (w/v) Na₂HPO₄ and 0.1% (w/v) KNO₃, was sterilized by autoclaving (15 psi, 121°C, 15 min). The complete defined medium was produced according to Haslam *et al.* (12) and sterilized by membrane filtration (0.22 µm). The medium (5 ml) was seeded with colonies prior to incubation at 37°C in an anaerobic chamber for 4 days and the supernatants were then collected by centrifugation. TcdA and TcdB were quantitatively measured by ELISA. A concentration of <0.005 µg was recorded as negative (12).

Results and Discussion

Ribotyping and toxinotyping of isolates. Of the 325 stool samples, 40 isolates (12%) were identified as *C. difficile*-positive. The prevalence was significantly higher compared to that in a previous study by Huang *et al.* (15). As listed in Table I, 18 isolates were collected from the Physical Examination Department (DPE), 13 from the Clinical Laboratory Department (DCL) and 9 from the Clinical Microbiology Department (DCM). The *C. difficile* ribotypes were identified based on the PCR targeting to the 16S-23S rRNA gene intergenic spacer region. Among the 40 isolates, 4 different PCR-ribotypes (001, 002, 006 and 014) were identified and ribotype 006 accounted for 55% of the cases (22/40) and was therefore marked as the dominant ribotype. The ribotypes 002, 014 and 001 accounted for 30% (12/40), 10% (4/40) and 5% (2/40) of the cases, respectively. The results were not consistent with those of previous studies in China or other countries (15-17), confirming the geographical variance among the different ribotypes.

Immunochromatography and cytotoxicity assays were performed to determine the presence of TcdA and TcdB. The result demonstrated that the percentage of type A⁺B⁺ was 65% (26/40), whereas types A⁻B⁺ and A⁻B⁻ were 25% (10/40) and 10% (4/40), respectively. The role of the two toxins remains

Table I. Distribution of polymerase chain reaction (PCR) ribotypes and toxigenicity among *Clostridium difficile* strains isolated from different wards.

Isolates	PCR ribotype	Unit	Toxin test	
			TcdA (immunochromatography assay)	TcdB (cytotoxicity assay)
1	006	DPE	+	+
2	006	DPE	+	+
3	006	DPE	+	+
4	006	DPE	+	+
5	006	DPE	+	+
6	006	DPE	+	+
7	006	DPE	+	+
8	006	DPE	+	+
9	006	DPE	+	+
10	006	DCM	+	+
11	006	DCM	+	+
12	006	DCM	+	+
13	006	DCM	+	+
14	006	DPE	+	+
15	006	DPE	+	+
16	006	DPE	+	+
17	014	DPE	+	+
18	014	DPE	+	+
19	014	DPE	+	+
20	014	DPE	+	+
21	002	DCL	+	+
22	002	DCL	+	+
23	002	DCL	+	+
24	002	DCL	+	+
25	002	DCL	+	+
26	002	DCL	+	+
27	006	DPE	-	+
28	006	DPE	-	+
29	006	DCM	-	+
30	006	DCM	-	+
31	006	DCM	-	+
32	006	DCL	-	+
33	002	DCL	-	+
34	002	DCL	-	+
35	002	DCL	-	+
36	002	DCL	-	+
37	002	DCM	-	-
38	002	DCM	-	-
39	001	DCL	-	-
40	001	DCL	-	-

TcdA, enterotoxin A; TcdB, cytotoxin B; DPE, Physical Examination Department; DCM, Clinical Microbiology Department; DCL, Clinical Laboratory Department.

debated upon and type A⁻B⁺ has increased significantly over the last few years (18). However, type A⁺B⁺ is considered to play the most important role in eliciting CDI (19).

Table II. Toxin production by different *Clostridium difficile* strains in tryptic nitrate broth and complete defined medium.

Isolates	Toxin production in			
	Tryptic nitrate broth		Complete defined medium	
	TcdA ($\mu\text{g/ml}$)	TcdB (titer)	TcdA ($\mu\text{g/ml}$)	TcdB (titer)
1	0.14	0.03	0.04	0.15
2	0.18	0.08	0.05	0.02
3	0.36	0.15	0.12	0.37
4	3.5	0.19	1.10	5.60
5	18.0	2.30	0.32	0.5
6	0.62	0.60	0.16	0.92
7	0.73	0.28	0.24	0.42
8	0.88	0.34	0.37	0.02
9	1.95	1.50	3.10	0.09
10	15.5	1.60	0.13	7.80
11	3.53	2.27	0.04	0.42
12	0.24	0.11	0.22	4.30
13	0.88	0.23	0.19	1.40
14	0.23	0.05	0.06	0.07
15	0.19	0.18	0.64	0.23
16	3.2	0.27	0.98	1.23
17	3.9	0.34	2.4	1.34
18	0.94	1.44	0.56	0.59
19	3.88	3.45	0.09	3.22
20	2.97	2.31	0.18	0.45
21	0.98	2.56	0.77	2.78
22	4.32	3.42	3.96	0.09
23	0.96	0.78	0.55	3.42
24	2.55	2.31	0.15	1.54
25	2.44	2.56	0.98	0.87
26	3.56	3.06	0.45	2.37
27	0	0.41	0	0.02
28	0	5.10	0	0
29	0	6.30	0	0.65
30	0	0.36	0	2.37
31	0	0.49	0	0
32	0	2.90	0	0
33	0	3.10	0	0.46
34	0	0.87	0	0.34
35	0	3.12	0	2.35
36	0	1.34	0	0.45
37	0	0	0	0
38	0	0	0	0
39	0	0	0	0
40	0	0	0	0

TcdA, enterotoxin A; TcdB, cytotoxin B.

Among the ribotype 006 isolates, 16 out of 22 (54.5%) were toxin type A⁺B⁺ and the remaining were A⁻B⁺. In the 12 ribotype 002 isolates, the percentage of types A⁺B⁺, A⁻B⁺

and A⁻B⁻ was 50% (6/12), 33.3% (4/12) and 16.7% (2/12), respectively. All 4 ribotype 014 isolates were A⁺B⁺ and the 2 ribotype 001 isolates were A⁻B⁻. These results suggest that the PCR ribotype may be associated with the toxigenic status. Similar results were reported by van den Berg *et al* (20), who demonstrated that all the A⁺B⁺ *C. difficile* strains from Poland belonged to the ribotype 017. However, the correlation between ribotypes and toxin types requires further confirmation by data derived from a large number of isolates.

Production of toxins in TNB and complete defined media.

To investigate the nutritional dependence of the production of TcdA and TcdB, ELISA was performed to quantify these two toxins using anti-toxin A or anti-toxin B monoclonal antibodies. The production of the toxins by the 40 *C. difficile* isolates in TNB and complete defined media is summarized in Table II. The composition of the media significantly affected the growth of the three *C. difficile* strains and the production of TcdA and TcdB. This result is consistent with the studies reported by Haslam *et al* (12) and Osgood *et al* (11).

In the present study, the production of TcdA by the 40 isolates was generally higher compared to that of TcdB in the TNB medium. The majority of the isolates exhibited a higher TcdA production in TNB compared to that in complete defined medium. However, compared to that of TcdA, the concentration of TcdB depended on the strains as well as the medium in which the isolates were grown. Some strains produced a high amount of TcdB when grown in TNB, whereas others exhibited higher production in the complete defined medium. This may be explained by the strict regulation imposed by environmental factors and different regulators on TcdB synthesis by different strains. The highest TcdA production was observed in isolate 4 when it was grown in TNB (18.0 $\mu\text{g/ml}$). Furthermore, the highest TcdB concentration was produced by isolate 10, when it was grown in the complete defined medium (7.80 titer).

It was previously reported that TcdA was detected in isolates that could not produce TcdB and the A⁻ isolates produced a large amount of TcdB (20). Our results also suggested that TcdA and TcdB are independently produced. Thus, the clinical diagnosis of *C. difficile* should focus on the detection of both TcdA and TcdB, particularly in the A⁻B⁺ strains being isolated with increasing frequency. Consistent with the immunochromatography and cytotoxicity assay results, no production of TcdA by isolates 27-40 or of TcdB by isolates 37-40 was detected in either medium. However, for isolates 28, 31 and 32, which were TcdB⁺ according to the cytotoxicity assay, TcdB production was detected in the TNB medium, proving that the synthesis of this toxin largely relies on the composition of the medium.

In conclusion, in this study, 4 ribotypes of *C. difficile* were identified and the ability of these ribotypes to produce TcdA and TcdB appears to be affected by the nutritional status. This finding is potentially useful in the clinical diagnosis and prevention of CDI.

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