

Investigation of Toxin Gene Diversity, Molecular Epidemiology, and Antimicrobial Resistance of *Clostridium difficile* Isolated from 12 Hospitals in South Korea

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Background: *Clostridium difficile* is a major cause of antibiotic-associated diarrhea. The objective of this study was to characterize clinical isolates of *C. difficile* obtained from various regions in Korea with regard to their toxin status, molecular type, and antimicrobial susceptibility.

Methods: We analyzed a total of 408 *C. difficile* isolates obtained between 2006 and 2008 from 408 patients with diarrhea in 12 South Korean teaching hospitals. *C. difficile* toxin genes *tcdA*, *tcdB*, *cdtA*, and *cdtB* were detected by PCR. Molecular genotyping was performed by PCR ribotyping. Antimicrobial susceptibilities of the 120 *C. difficile* isolates were assessed by agar dilution methods.

Results: Among 337 toxigenic isolates, 105 were toxin A-negative and toxin B-positive (AB⁺) and 29 were binary toxin-producing strains. PCR ribotyping showed 50 different ribotype patterns. The 5 most frequently occurring ribotypes comprised 62.0% of all identified ribotypes. No isolate was susceptible to cefoxitin, and all except 1 were susceptible to piperacillin and piperacillin-tazobactam. The resistance rates of isolates to imipenem, cefotetan, moxifloxacin, ampicillin, and clindamycin were 25%, 34%, 42%, 51%, and 60%, respectively. The isolates showed no resistance to metronidazole or vancomycin.

Conclusions: This is the first nationwide study on the toxin status, including PCR ribotyping and antimicrobial resistance, of *C. difficile* isolates in Korea. The prevalence of AB⁺ strains was 25.7%, much higher than that reported from other countries. Binary toxin-producing strains accounted for 7.1% of all strains, which was not rare in Korea. The most prevalent ribotype was ribotype 017, and all AB⁺ strains showed this pattern. We did not isolate strains with decreased susceptibility to metronidazole or vancomycin. (*Korean J Lab Med* 2010;30:491-7)

Key Words : *Clostridium difficile*, toxin A, toxin B, Ribotyping, Drug Resistance, Epidemiology

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INTRODUCTION

Clostridium difficile is a major cause of nosocomial antibiotic-associated diarrhea [1]. Toxins A (enterotoxin; TcdA) and B (cytotoxin; TcdB) are well-known primary virulence factors of *C. difficile* [2]. These toxins are encoded by 2 separate genes, *tcdA* and *tcdB*, which are located

in the pathogenicity locus of the chromosome called PaLoc [3, 4].

Toxigenic isolates of *C. difficile* usually produce both toxins A and B. Toxin A-negative and toxin B-positive (A⁻B⁺) strains of *C. difficile* were first described in the early 1990s [5,6]. A⁻B⁺ strains fail to produce detectable amounts of toxin A due to a deletion in the repeating sequence of the *tcdA* gene. However, A⁻B⁺ strains have been associated with clinical conditions ranging from asymptomatic carriage to fatal pseudomembranous colitis. Alfa et al. [7] reported convincing evidence that indicates that these strains have been responsible for outbreaks in hospitals.

Some isolates of *C. difficile* produce an additional binary toxin (actin-specific ADP-ribosyltransferase toxin, CDT), whose role in *C. difficile*-associated disease (CDAD) is unclear [8]. The 2 genes *cdtA* and *cdtB* encode the enzymatic (CDTa) and binding (CDTb) components of the binary toxin. These genes are located on the CDT locus of the chromosome but are not part of the PaLoc [8, 9]. The prevalence of A⁻B⁺, and binary toxin-producing *C. difficile* strains varies geographically [10].

Recently, outbreaks of CDAD due to an emerging strain of *C. difficile* (PCR ribotype 027) associated with high morbidity and mortality have been reported in Canada, the United States, and Europe [11]. This strain produces a binary toxin and has deletions in *tcdC*, a putative negative regulator for toxins A and B [11, 12]. The epidemic strain is resistant to gatifloxacin and moxifloxacin, and increasing use of fluoroquinolone has been considered a risk factor in these outbreaks [11]. The most commonly used drugs for the treatment of CDAD are metronidazole (MTZ) and vancomycin (VAN). *C. difficile* is considered to be susceptible to both agents, and therefore, the *in vitro* activity of these agents against *C. difficile* isolates is rarely performed in most centers. However, a few reports have been published regarding elevated minimum inhibitory concentrations (MIC) of MTZ and VAN against *C. difficile* [13]. Moreover, increased resistance to antimicrobial agents has played a role in their selection in hospital environments [14].

The objective of this study was to characterize clinical

isolates of *C. difficile* associated with diarrhea throughout South Korea with regard to their toxin status, molecular typing, and antimicrobial susceptibility.

MATERIALS AND METHODS

1. Bacterial strains

We obtained and analyzed 408 unduplicated isolates of *C. difficile* recovered between 2006 and 2008 from 408 patients with diarrhea in 12 tertiary teaching hospitals in 7 regions of Korea. We received *C. difficile* isolates or frozen stool samples from all 12 hospitals. Stool samples were cultured anaerobically on *C. difficile* selective agar (CDSA, Becton Dickinson and Company, Sparks, MD, USA) for 48 hr at 37°C. Species identification was performed on the basis of typical morphology on agar plates as well as characteristic odor and ATB 32A system results (BioMerieux SA, Marcy l'Etoile, France). The reference strains VPI 10463, 3608/03, SE844, 48489, 1470, and UK078 were supplied by Dr. Maja Rupnik, Michel Delmee, and Thomas V. Riley.

2. Toxin analysis by PCR

C. difficile toxin genes were detected by PCR as described previously [15, 16]. The primer pairs used were NK9-NK11 for the repetitive domain of *tcdA*, NK104-NK105 for *tcdB*, *cdtA* pos-*cdtA* rev for *cdtA*, and *cdtB* pos-*cdtB* rev for *cdtB*.

3. PCR ribotyping

PCR ribotyping was performed as previously described with the primers 5'-CTGGGGTGAAGTCGTAACAAGG-3' (position 1445 to 1466 of the 16S rRNA gene) and 5'-GCGCCCTTTGTAGCTTGACC-3' (position 20 to 1 of the 23S rRNA gene) [17]. Comparison of the PCR ribotyping patterns was performed visually. Ribotype patterns that differed by at least 1 band were assigned to different types. Ribotype groups were designated by upper- and

lower-case letters combined with a number.

4. *tcdC* sequencing

The *tcdC* gene was PCR-amplified with the primers PaL15 and PaL16 on the ribotype 027 strain as previously described [18]. Amplicons were sequenced commercially (Macrogen, Seoul, Korea). The analyzed amino acid sequences were compared to the published *tcdC* sequence for strain VPI10463 [18].

5. Antimicrobial susceptibility testing

Antimicrobial susceptibility tests were performed with 120 *C. difficile* isolates using 10 randomly selected isolates per hospital and the agar dilution method on Brucella blood agar according to the recommendations of the CLSI [19]. Quality control strains used for susceptibility testing included *Bacteroides thetaiotaomicron* (ATCC 29741) and *B. fragilis* (ATCC 25285). Antimicrobial agents used were ampicillin (Sigma-Aldrich Co., St. Louis, MO, USA), piperacillin and tazobactam (Yuhan, Seoul, Korea), cefoxitin (Merck Sharp & Dohme, West Point, PA, USA), cefotetan (Daiichi Pharmaceutical, Tokyo, Japan), clindamycin (Korea Upjohn, Seoul, Korea), imipenem and

metronidazole (Choong Wae, Seoul, Korea), moxifloxacin (Bayer Korea, Seoul, Korea), and vancomycin (Chong Kun Dang, Seoul, Korea). For the combination of piperacillin and tazobactam, a constant amount of tazobactam (final concentration, 4 $\mu\text{g}/\text{mL}$) was added to piperacillin. The CLSI breakpoints were used for the analysis. However, the CLSI guidelines do not recommend a breakpoint for VAN, and therefore the breakpoint suggested by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.escmid.org/research_projects/eucast) was used.

RESULTS

1. Toxin analysis by PCR

Of the total 408 isolates, 337 (82.6%) were toxigenic *C. difficile* (A⁺B⁺ and A⁻B⁺). We identified 232 (56.9%) A⁺B⁺ strains and 105 (25.7%) A⁻B⁺ strains. The recovery rates of the toxigenic strains were 70–100% according to the hospitals studied. The proportion of A⁻B⁺ strains differed between the hospitals during the study period (from 0% to 37.9%).

Twenty-nine (7.1%) strains were CDT⁺. The proportion of CDT⁺ strains varied between the hospitals (from 0% to

Table 1. The frequencies of the occurrences of toxins A-, B-, and binary toxin-producing strains in 12 South Korean hospitals

Hospitals	N of isolates tested	Study period	Number of beds*	N (%)			
				A ⁺ B ⁺ CDT ⁻	A ⁺ B ⁺ CDT ⁺	A ⁻ B ⁺ CDT ⁻	A ⁻ B ⁺ CDT ⁺
Seoul A	145	Jan.2007-Dec. 2007	2,064	52 (35.9)	8 (5.5)	55 (37.9)	30 (20.7)
Seoul B	37	Jan.2007-Jun.2008	758	19 (51.4)	4 (10.8)	9 (24.3)	5 (13.5)
Seoul C	30	Jan.2007-Feb.2008	938	13 (43.3)	6 (20.0)	6 (20.0)	5 (16.7)
Seoul D	20	Jan.2008-Feb.2008	2,200	8 (40.0)	3 (15.0)	3 (15.0)	6 (30.0)
Gyeonggi A	41	Jun.2007-Mar.2008	589	23 (56.1)	2 (4.9)	9 (21.9)	7 (17.1)
Gyeonggi B	17	Jan. 2008-May. 2008	920	14 (82.3)	1 (5.9)	2 (11.8)	0 (0)
Gyeonggi C	15	Jan.2006-Dec. 2006	550	8 (53.3)	0 (0)	3 (20.0)	4 (26.7)
Chungnam	22	Oct.2007-May.2008	803	12 (54.5)	2 (9.1)	6 (27.3)	2 (9.1)
Daejeon	25	Mar.2008-Jun.2008	813	17 (68.0)	2 (8.0)	3 (12.0)	3 (12.0)
Busan	20	Feb.2007-Dec. 2007	912	12 (60.0)	0 (0)	6 (30.0)	2 (10.0)
Gwangju	20	Mar.2008-Jun.-2008	555	15 (75.0)	0 (0)	0 (0)	5 (25.0)
Gangwon	16	Nov.2007-May.2008	816	10 (62.5)	1 (6.3)	3 (18.8)	2 (12.5)
Total	408			203 (56.9)	29 (7.1)	105 (25.7)	71 (17.4)

*data from the Korean Hospital Association 2007.

Abbreviations: A⁺B⁺, toxin A-positive, toxin B-positive; A⁻B⁺, toxin A-negative, toxin B-positive; A⁻B⁻, toxin A-negative, toxin B-negative, CDT⁺, binary toxin-positive; CDT⁻, binary toxin-negative.

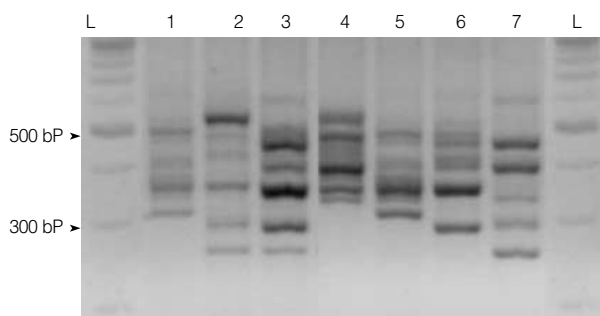


Fig. 1. PCR ribotype patterns of the *Clostridium difficile* isolates representing PCR ribotypes AB24, C11, C5, C2, AB14, AB23, and aB (Lane 1 to 7, respectively). Lane L refers to 100 bp ladder. Banding patterns of the C5, C2, and aB ribotypes were identical to the pattern of *C. difficile* ribotype 027, 078, and 017 strains.

20.0%). All CDT⁺ strains were A⁺B⁺ (Table 1).

2. PCR ribotyping

A total of 50 different ribotype patterns were found. We identified 24 patterns of A⁺B⁺ CDT⁻ strains (ribotype AB1-AB24), 12 A⁺B⁺ CDT⁺ (ribotype C1-C12), and 13 A⁻B⁻ (ribotype ab1-ab13). The PCR ribotypes aB, C5, and C2 are equivalent to the PCR ribotypes 017, 027, and 078 by O'Neill's method, respectively [17] (Fig. 1).

All A⁻B⁻ strains showed the same banding pattern (ribotype aB) in ribotyping, which was identical to the pattern of the *C. difficile* 1470 strain (ribotype 017). Ribotype aB was the predominant type (105 isolates, 25.7%). The most frequently observed ribotypes of *C. difficile* in decreasing order were as follows: ribotype AB2 (71, 17.4%), ribotype AB3 (30, 7.4%), ribotype AB1 (27, 6.6%), and ribotype AB17 (20, 4.9%). These 5 ribotypes comprised 62.0% of the total. Ribotypes of *C. difficile* isolates were different from their toxin statuses.

Only 1 *C. difficile* isolate showed a pattern (ribotype C5) identical to PCR ribotype 027. Sequence analysis of *tcdC* in this isolate showed a single-base pair deletion at position 117 as well as a well-documented 18-bp deletion, which was identical to the sequence results of the epidemic strain of *C. difficile* 027. When examined by the E-test, the isolate was susceptible to moxifloxacin (MIC=0.5 µg/mL).

Thirteen strains of PCR ribotype 078 (ribotype C2) were identified in 6 hospitals, making ribotype 078 the most

Table 2. The MICs of 10 antimicrobial agents for 120 Korean *Clostridium difficile* isolates

Antimicrobials	MIC (µg/mL)			S	I	R
	Range	MIC ₅₀	MIC ₉₀			
Ampicillin	1-8	2	2	0	49	51
Piperacillin	2-64	8	16	99	1	0
Piperacillin-tazobactam	4-64	8	16	99	1	0
Cefoxitin	64->128	128	>128	0	0	100
Cefotetan	8->128	32	128	26	40	34
Clindamycin	0.5->128	128	>128	11	29	60
Imipenem	2-64	8	16	22	53	25
Metronidazole	0.12-8	1	4	100	0	0
Moxifloxacin	1->128	2	16	53	5	42
Vancomycin	0.25-2	0.5	1	100	0	0

Abbreviations: MIC, minimum inhibitory concentration; S, Susceptible; I, Intermediate; R, Resistant.

prevalent ribotype among CDT⁺ strains (13/29 CDT⁺ strains, 44.8%; 13/408 isolates, 3.1%).

3. Antimicrobial susceptibility testing

The *in vitro* activities of antimicrobial agents against *C. difficile* isolates are summarized in Table 2. No isolates were susceptible to cefoxitin and all except 1 were susceptible to piperacillin and piperacillin-tazobactam. The resistance rates to imipenem, cefotetan, moxifloxacin, ampicillin, and clindamycin were 25%, 34%, 42%, 51%, and 60%, respectively. All strains were susceptible to metronidazole and vancomycin.

DISCUSSION

We conducted this study to enhance the knowledge on the nationwide epidemiology of *C. difficile*. This study included data from 12 hospitals in 7 different areas of South Korea.

The prevalence of A⁻B⁻ strains differs according to the country studied. In Europe, 6.2% of toxigenic *C. difficile* isolates recovered in 2005 were A⁻B⁻ [10]. In a recent study, A⁻B⁻ strains comprised 33.3% of 75 toxigenic isolates from Shanghai and 0% of 80 isolates from Stockholm [20]. The prevalence of A⁻B⁻ strains was 25.7% (0-37.9%, according to the data obtained from hospitals) in

this study. In our previous study, the prevalence of A⁻B⁺ strains increased steadily (4.2% in 1995, 39.6% in 2004) [21], and in another multicenter study conducted in Korea, 17.6–54.8% of the isolated strains were A⁻B⁺ in 2005 [22]. The prevalence of A⁻B⁺ strains in Korea and Shanghai was much higher than in European countries.

The prevalence of CDT⁺ strains was 7.1% (0–20.0%) in this study. Before the epidemics caused by ribotype 027, a binary toxin was identified in about 6% of clinical *C. difficile* isolates obtained in the United States and Europe [16, 23]. The prevalence of CDT⁺ *C. difficile* strains increased to 34.6% due to the ribotype 027 epidemics in Canada [24]. In our previous study, the prevalence of CDT⁺ strains increased from 0% in 2003 to 3.9% in 2006 [21]. Therefore, we thought the prevalence of CDT⁺ strains had steadily increased without evidence of a *C. difficile* epidemic. All CDT⁺ strains were A⁺B⁺. Therefore, no additional binary toxin test was required for the diagnosis of CDAD.

A total of 408 *C. difficile* isolates were successfully typed with our PCR ribotyping method. Predominant ribotypes among the participating hospitals were not significantly different.

All 105 A⁻B⁺ strains showed the same ribotyping pattern (aB), which was the most common ribotype (105/408, 25.7%) and indistinguishable from the pattern of *C. difficile* 1470 (ribotype 017). It was previously reported that most A⁻B⁺ strains yield this distinct ribotype pattern in many studies, suggesting a worldwide clonal spread [7, 10, 21].

Only 1 PCR ribotype 027 strain was identified in hospital Seoul A. In contrast to epidemic 027 strains resistant to fluoroquinolone, this isolate was susceptible, which is in accordance with a report on 027 isolates obtained before 2001 in North America [11].

PCR ribotype 078 is the predominant ribotype in calves and pigs, and is an emerging new hypervirulent strain [25]. The prevalence of CDAD caused by a PCR ribotype 078 strain increased from 3% to 13% during 2005–2008 in The Netherlands. CDAD caused by type 078 strains has a similar severity of CDAD caused by type 027 strains [26]. Thirteen strains of PCR ribotype 078 were identi-

fied in our study, which was the most prevalent ribotype among CDT⁺ strains (44.8% of CDT⁺ strains, 3.1% of all isolates).

Antimicrobial therapy plays a central role in the development of CDAD. The increasing use of fluoroquinolones in US health care facilities may have provided a selective advantage for the fluoroquinolone-resistant 027 strain and promoted its widespread emergence [11]. MTZ and VAN remain the most active agents in this study. No resistance to piperacillin-tazobactam was found in isolates from Shanghai and Stockholm [20] and only 1 non-toxigenic isolate showed intermediate resistance in this study. Resistance to other antimicrobials varies widely between countries [29]. The resistance rate to moxifloxacin was 42% in our study, which was lower than that in Scotland (87.5%, 2007) and higher than that in Sweden (15.0%, 2009). The resistance rate to clindamycin was 60% in our study, which was lower than in Canada (90.9%, 2009) and higher than that in Hungary (27.5%, 2009) [27].

The MICs of ampicillin, piperacillin, piperacillin-tazobactam, cefoxitin, cefotetan, imipenem, metronidazole, and vancomycin were not significantly different according to the toxin status. However, the MIC₅₀ values of clindamycin and moxifloxacin in A⁻B⁺ strains were significantly higher than those of A⁺B⁺ strains: 128 and 16 in A⁻B⁺ versus 4 and 1 in A⁺B⁺, respectively (data not shown). It was reported that higher MICs of antimicrobial agents for predominant *C. difficile* strains may have played a role in their persistence and dissemination in hospitals [28, 29]. Therefore, the increased prevalence of A⁻B⁺ strains in this study may reflect their higher MICs and the selective advantage it allows. This is the first nationwide study on the toxigenic status, including molecular genotyping and antimicrobial susceptibility pattern, of *C. difficile* isolates in South Korea. The prevalence of A⁻B⁺ and CDT⁺ strains was 25.7% and 7.1%, respectively. Surveys of all A⁻B⁺ strains showed that the most common ribotype was ribotype 017. We isolated 1 ribotype 027 strain, which is regarded as a historic isolate, with susceptibility to moxifloxacin. The prevalence of ribotype 078 was 3.1%, which was higher than that of

ribotype 027. We did not isolate strains with decreased susceptibility to MTZ or VAN, since these 2 antimicrobial agents can be used without an antimicrobial susceptibility test.

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