ORIGINAL ARTICLE

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Characterization of *Clostridium difficile* Strains Isolated from Patients with *C. difficile*-associated Disease in Korea

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Objectives: Studies on *Clostridium difficile* are rare in Korea. We investigated the epidemiological characteristics of *C. difficile* isolates from patients with *C. difficile*-associated disease (CDAD) in Korea.
Methods: Multiplex polymerase chain reaction was performed to detect the presence of *tcdA* and *tcdB* toxin genes. Antimicrobial susceptibility test was carried out by the disk-dilution method. *C. difficile* strains were subtyped by automated repetitive-element palindromic PCR (rep-PCR).
Results: Among patients with CDAD, 73 (25.8%), 32 (11.3%), 32 (11.3%), and 26 (9.2%) suffered from pneumonia, cancer or neoplasm, diabetes, and colitis, respectively. Of all stool samples, 43 samples (15.2%) were positive for *C. difficile* strains. We observed two expression patterns of toxin genes: *tcdA*+/*tcdB*+ (86% isolates) and *tcdA*−/*tcdB*+ (14% isolates), with all isolates expressing *tcdB*. Furthermore, some isolates were resistant to clindamycin (65%), ampicillin (56%), and cefazolin (40%), but all were susceptible to vancomycin and metronidazole. The tested samples were classified into diverse clusters using automated rep-PCR.
Conclusion: Our findings revealed the characteristics and antibiotic resistance of *C. difficile* isolates from patients in Korea. The epidemiological data may provide valuable insight into development of treatment strategies for *C. difficile* infections in Korea.

Key Words: *Clostridium difficile*, epidemiological characterization

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INTRODUCTION

Since 1978, *Clostridium difficile* has been known to cause pseudomembranous colitis (PMC) through the release of toxins, and infection with this pathogen is associated with antibiotic use in patients with diarrhea [1]. The two major *C. difficile* toxins include a 308 kDa enterotoxin (toxin A, *tcdA*) and 270 kDa cytotoxin (toxin B, *tcdB*), which constitute important pathogenic factors associated with colitis [2]. These toxins destroy the cytoskeleton of intestinal cells, in-

activate proteins, damage cells and secretions of intestinal fluid, and induce inflammation and apoptosis. The symptoms associated with *C. difficile* infections (CDIs) are due to the expression of *tcdA* and *tcdB* genes. CDIs may be classified as mild with self-limiting diarrhea or severe with complications due to excessive infection [3]. Although the intake of excessive antibiotics is the main cause of infectious diarrhea, including CDI, CDI was also reported in low-risk groups of patients not using antibiotics. *C. difficile*-associated disease (CDAD) causes watery or mucous-containing stools accompanied with abdominal pain and fever [4]. The frequency of CDAD increases in low-risk groups and is related to functions associated with proton-pump inhibitors (PPIs) [5]. The activity of PPIs may increase the pH in the stomach, which may speed up *C. difficile* propagation in a repressed state [6,7].

In recent years, the incidence of CDI has continuously increased in Europe and the United States and *C. difficile* is reported to be an important source of infection [8]. According to a domestic research literature related to CDI and PMC from 1988 to 1997 and single-agency data from 2000 to 2005, the frequency of infection during this period was not high [9]. However, CDAD incidence and severe illness in patients hospitalized between 2004 and 2008 was 1.7/1,000 and 2.7/1,000 people, respectively, as indicated in another study focusing on 17 university hospitals around Korea [10]. In this period, > 60% of 1,367 patients with CDAD were prescribed metronidazole during the initial treatment; only an 8.9% relapse rate and a 3.6% rate of severe illness and complications were reported. During this time, Korea exhibited fewer clinical manifestations of CDAD with better as compared with cases reported in Europe and the United States; however, the incidence rate continued to gradually increase [11]. Therefore, a survey of *tcdA* and *tcdB* genes from *C. difficile* isolated from patients with diarrhea and other diseases is required along with the epidemiological research comparing the information from these patients with the characteristics of *C. difficile* isolates. However, studies regarding *C. difficile* characteristics in patients with CDI in Korea are insufficient.

In this study, we isolated *C. difficile* from patients and identified its toxin types, spore formation, and antibiotic susceptibility to compare and correlate these findings with other diseases found in the patient cohort. Moreover, we identified *C. difficile* lineages by performing repetitive element palindromic (rep)-polymerase chain reaction (PCR) and phylogenetic analyses on *C. difficile* strains isolated from patients admitted in a clinic in Korea.

MATERIALS AND METHODS

1. Collection of stool samples according to disease symptoms

Stool samples were collected from 283 patients with diarrhea who were tested for CDI from April 2013 to October 2013 at a clinic in Korea. This study was approved by the Institutional Review Board of the Bundang Jesaeng General Hospital (LAB15-01). Medical records of the patients enrolled in this study were reviewed by performing an epidemiological analysis of age- and gender-specific patterns, duration of hospitalization, symptoms, and antibiotics used, to prevent patient duplication. Moreover, leukocyte number, total protein level, albumin level, bilirubin level, C-reactive protein (CRP) level, and erythrocyte sedimentation rate (ESR) were measured using blood assays.

2. Identification of bacterial isolates

Stool samples were gently mixed with 95% (v/v) ethanol at 1:1 ratio and incubated for 1 hour at 25°C. The mixture was inoculated on ChromID *C. difficile* selective medium (bioMérieux, Marcy l'Etoile, France) and incubated under anaerobic conditions (Bugbox; Ruskinn Technology, Bridgend, UK) for 48 to 72 hours at 37°C. Black-colored colonies were inoculated on blood agar plates (BAPs) and incubated under anaerobic conditions for 48 to 72 hours at 37°C. An API rapid ID 32A system kit (bioMérieux) was used for *C. difficile* identification according to the manufacturer's instructions.

3. PCR for toxin genes

Isolated colonies grown on BAPs were analyzed by multiplex PCR for the presence of *tcdA* and *tcdB*. DNA was extracted using QIAamp DNA mini kit (51306; Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR was performed using a PerkinElmer thermal cycler PCR system 2400 (PerkinElmer, Waltham, MA, USA). The primers used for the detection of *tcdA* and *tcdB* were as follows: *tcdA* forward, 5'-AGA TTC CTA TAT TTA CAT GAC AAT AT-3'; *tcdA* reverse, 5'-GTA TCA GGC ATA AAG TAA TAT ACT TT-3'; and *tcdB* forward, 5'-GGA AAA GAG AAT GGT TTT ATT AA-3'; *tcdB* reverse, 5'-ATC TTT AGT TAT AAC TTT GAC ATC TTT-3'. The reaction conditions were as follows: initial denaturation at 95°C for 15 minutes, 30 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 2 minutes, extension at 72°C for 2 minutes, and final extension at 72°C for 5 minutes. The PCR product was subjected to gel electrophoresis using 1% agarose, followed by staining with ethidium bromide and visualization under an ultraviolet trans-illuminator.

4. Microscopic analysis

For determining spores in the isolates, *C. difficile* strains positive for *tcdA/B* were directly examined by electron microscopy. The bacterial cells were diluted in 1.5 mL of 0.85% saline and washed thrice at 12,000 rpm. The bacterial sample was fixed in 2.5% glutaraldehyde/0.1 M phosphate-buffered saline (PBS; pH 7.4) for 2 hours and then transferred to 1% osmium tetroxide for 1 hour. The sample was washed with 0.1 M PBS, embedded into epoxy resin using propylene oxide, and dehydrated using alcohol. After embedding, the sample was polymerized in a dry oven at 60°C for 12 hours. In the subsequent steps, the bacteria-embedded resin was cut into 70 to 80 mm thick sections using an ultramicrotome (Ultra cut C; Leica, Wetzlar, Germany) and observed under an electron microscope (Cryo-TEM CryoTecnai F20; FEI, Hillsboro, OR, USA) after staining with uranyl acetate and lead citrate. Two standard strains, ATCC-43598 (*tcdA*−/*tcdB*+) and KCCH-12115 (*tcdA*+/*tcdB*+), were used for the comparison of spores.

5. Antimicrobial susceptibility

The antimicrobial susceptibility of *C. difficile* isolates was evaluated by the disk-dilution method using ampicillin, cefazolin, imipenem, amikacin, clindamycin, and metronidazole. The E-test (bioMérieux) method for evaluating vancomycin resistance was also performed according to the guidelines of the Clinical and Laboratory Standards Institute.

6. Rep-PCR analysis

Twelve randomly selected isolates, including two reference strains, were used for molecular subtyping. DNA was extracted from each strain using a commercially available DNA extraction kit (UltraClean microbial DNA isolation kit; MoBio Laboratories, Solana Beach, CA, USA) according to manufacturer's instructions. Extracted DNA samples were quantified using a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA), followed by their amplification for DNA fingerprinting. The PCR reaction contained 2 μL of extracted genomic DNA (~25 ng/μL) and 23 μL of PCR mixture containing 0.5 μL (or 2.5 U) AmpliTaq polymerase (Applied Biosystems, Foster City, CA, USA), 2.5 μL 10× GeneAMP PCR buffer I (Applied Biosystems), 2 μL primer mix, and 18 μL rep-PCR master mix from the DiversiLab *Clostridium* kit (bioMérieux). Amplification was performed under following conditions: initial denaturation at 94°C for 2 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 70°C for 90 secodns, and final extension at 70°C for 3 minutes. PCR products were separated and analyzed using a microfluidic chip and an Agilent model 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). All tested samples were automatically analyzed by the Extended Jaccard coefficient using DiversiLab™ software (v2.1.66; bioMérieux). Dendrograms were created by determining the distance matrices and using the unweighted pair-group method with arithmetic mean. Banding patterns exhibiting high degrees of similarity (cut-off value, 98%) were considered indistinguishable.

RESULTS

1. Patient profiles

As shown in Table 1, there were more females than males (54.4% vs. 45.6%, respectively) among 283 patients enrolled. The average age was 64 ± 21 years; 66 patients (23.3%) aged between 70 and 79 years and 62 (22.9%) patients, between 80 and 89 years. The average duration of hospitalization was 30 ± 56 days and diarrhea occurrence was reported at 20.4 days after antibiotic administration. Among patients with CDAD, 73 (25.8%) also suffered from pneumonia, 32 (11.3%) from cancer or neoplasm, 32 (11.3%) from diabetes, and 26 (9.2%) from colitis (Table 2). The results of the hematological examination are shown in Table 3. In particular, parameters related to inflammation, such as white blood cell (WBC) count, ESR, and CRP level, were higher than other factors. A high percentage of patients used antibiotics

Table 1. Baseline characteristics with outcomes among 283 patients

Parameter	Data
Mean age (y)	64 ± 21 (1–96)
Age distribution (y)	
0–10	11 (3.9)
11–20	14 (4.9)
21–30	14 (4.9)
31–40	18 (6.4)
41–50	42 (14.8)
51–60	42 (14.8)
61–70	66 (23.3)
71–80	62 (21.9)
81–90	14 (4.9)
Sex (male:female)	130:153 (1:1.17)
Onset of diarrhea since antibiotics usage (day)	20 ± 18 (1–65)
Hospitalization (day)	30 ± 56 (3–580)

Values are presented as mean ± standard deviation (range) or number (%).

Table 2. Underlying diseases of 283 patients

Underlying disease	Number of patients (%)
Pneumonia	73 (25.8)
Cancer, neoplasm	32 (11.3)
Diabetes	32 (11.3)
Colitis	26 (9.2)
Diarrhea	20 (7.1)
Cerebrovascular disease	19 (6.7)
Urinary tract infection	14 (4.9)
Gastritis	13 (4.6)
Cirrhosis of the liver	10 (3.5)
Fractures	10 (3.5)
Others (tuberculosis, dementia, etc.)	34 (12.1)

Table 3. Hematological parameters of 283 patients

Variable	Laboratory data
WBC ($\times 10^3/\mu L$)	11.7 ± 5.9 (4.9–42.2)
Total bilirubin (mg/dL)	1.5 ± 2.0 (0.11–10.65)
ESR (mm/h)	59.9 ± 31.8 (8–120)
CRP (mg/dL)	7.2 ± 7.4 (0.19–34.0)
Total protein (gm/dL)	6.6 ± 0.7 (5.1–8.1)
Albumin (gm/dL)	3.5 ± 0.5 (2.3–4.6)

Values are mean ± standard deviation (range).
WBC, white blood cell; ESR, erythrocyte sedimentation rate; CRP, C-reaction protein.

(92.9% [263/283 patients]); 88 patients were administered with cephalosporin series of antibiotics (33.4%), while 121 patients were treated with glycopeptides series of antibiotics (vancomycin, 67 [25.5%] and carbapenem, 54 [20.5%]; Table 4). Many patients used several series of antibiotics together; 22 patients used four series and 21 patients, three series.

2. Toxin gene patterns, spore determination, and antibiotic susceptibility of *C. difficile* isolates

Among 283 stool samples tested using ChromID *C. difficile* selective media, 43 samples (15.2%) were positive for *C. difficile* strains. In addition, two subtypes of *C. clostridioforme*—one subtype of *C. septicum* and three subtypes of *C. perfringens*—were isolated. The toxin genes (*tcdA* and *tcdB*) in *C. difficile* isolates were detected using PCR amplification and all isolates tested in this study showed the expression of *tcdB*; only 37 of 43 samples were detected positive for *tcdA* gene. We found two major expression patterns for the toxin genes: *tcdA*+/*tcdB*+ (37 *C. difficile* isolates; 86%) and *tcdA*−/*tcdB*+ (6 isolates; 14%). Electron microscopy results revealed that the spores of most *C. difficile* isolates were in their original form (Figure 1). As shown in Table 5, some isolates showed high resistance to clindamycin (65%), ampicillin (56%), and cefazolin (40%) but all were susceptible to vancomycin and metronidazole.

Table 4. Previously used antibiotics by patients

Antibiotic	Number of cases (%)
Cephalosporin	88 (33.4)
Glycopeptides (vancomycin)[a]	67 (25.5)
Carbapenem[b]	54 (20.4)
Penicillin[b]	23 (8.7)
Glycopeptides (teicoplanin)[b]	18 (6.7)
Amphotericin B[b]	5 (2.1)
Colistin[b]	4 (1.6)
Linezolid[b]	3 (1.2)
Metronidazole[a]	1 (0.4)

[a]Less than three series of antibiotics, 220/263; [b]more than three series of antibiotics, 43/263.

3. Rep-PCR analysis of *C. difficile* isolates

Ten representative *C. difficile* strains isolated from patients with different diseases and two reference strains were subtyped by automated rep-PCR. The dendrogram and computer-generated banding-pattern images are presented in Figure 2. All tested isolates were classified into various clusters with similarities ranging from 65.8% to 99.5%. With the exception of two strains (number 6 and 7), all tested *C. difficile* isolates appeared to be genetically unrelated. Most isolates carrying similar characteristics such as antibiotic resistance and toxin profiles exhibited distinguishable rep-PCR banding patterns, suggesting that automated rep-PCR successfully discriminated *C. difficile* strains isolated from the patients. Two strains (number 6 and 7) displayed identical banding patterns, with over 99% similarity.

DISCUSSION

C. difficile is an important bacterium that causes healthcare-associated infections (HAIs); therefore, it is important to report the sources of the disease for accurate diagnosis, given that *C. difficile* toxin causes diarrhea and PMC associated with antibiotic use [12]. CDI may be classified as mild (with self-limiting diarrhea and may not require treatment), moderate, or severe (with complications). In particular, severe CDI is defined as a

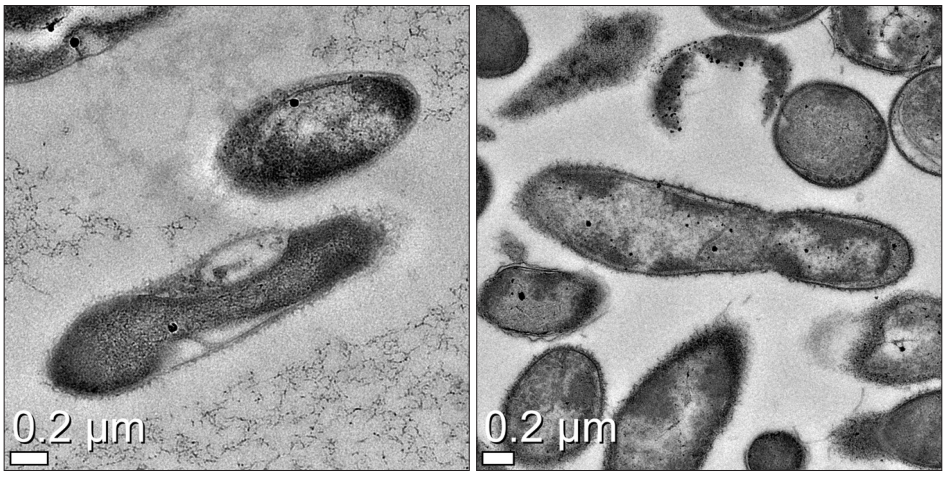

Figure 1. Observation of *Clostridium difficile* isolates under an electron microscope. (A) *C. difficile* isolates (*tcdA*−/*tcdB*+) and (B) (*tcdA*+/*tcdB*+).

Table 5. Antibiotic susceptibility of 43 *Clostridium difficile* isolates

Antimicrobial	Susceptibility	Intermediate	Resistance
Ampicillin (10 μg/mL)	0 (0)	19 (44)	24 (56)
Cefazolin (30 μg/mL)	14 (32)	12 (28)	17 (40)
Imipenem (10 μg/mL)	9 (20)	22 (50)	13 (30)
Amikacin (30 μg/mL)	34 (80)	9 (20)	0 (0)
Vancomycin (30 μg/mL)	43 (100)	0 (0)	0 (0)
Clindamycin (2 μg/mL)	4 (10)	11 (25)	28 (65)
Metronidazole (10 μg/mL)	43 (100)	0 (0)	0 (0)

Values are presented as number (%).

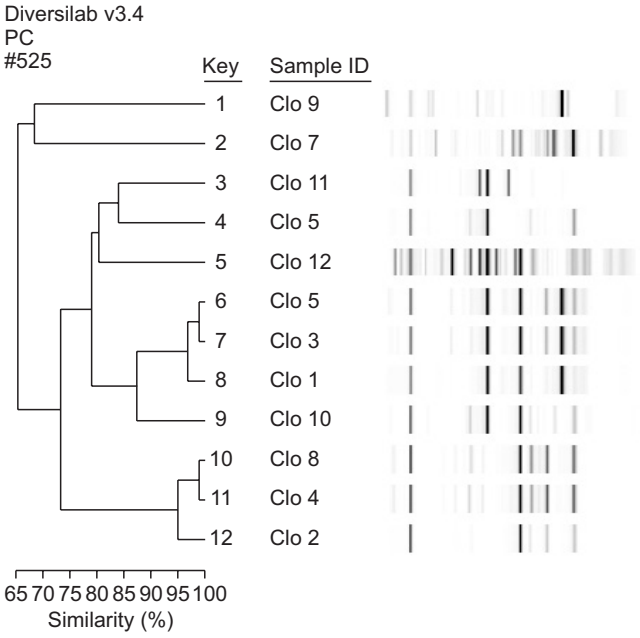

Figure 2. Repetitive-element palindromic-PCR dendrogram of *Clostridium difficile* isolates. Lanes 1, 2, and 10: *C. difficile* isolates (*tcdA*−/*tcdB*+); lanes 3, 4, and 5–9: *C. difficile* isolates (*tcdA*+/*tcdB*+); lane 11: ATCC-43598 (*tcdA*−/*tcdB*+); and lane 12: KCCH-12115 (*tcdA*+/*tcdB*+).

dangerous infection characterized by an increase in WBC count (> 15,000/mm^3) and in serum creatinine level (> 1.5-fold), and it may exhibit various complications such as lower blood pressure, shock, sepsis, intestinal paralysis, megacolon, gastrointestinal perforation, and death [3]. In Korea, the use of antibiotics and PPIs continues to increase, owing to inadequate restrictions related to the antibiotic use. A high proportion of patients (92.9%) in this study cohort was treated with antibiotics, and several antibiotic series were used together for treating many patients. The study cohort also included a higher proportion of older patients (aged 70–89 years) with weak immune systems. Hence, HAI caused by *C. difficile* are expected to be more frequent in Korea. As a result, there is an increased interest in the diagnosis of CDI. A previous study reported that rapid and accurate *C. difficile* diagnosis is critical for the treatment of patients and prevention of antibiotic abuse [13].

The expression pattern of toxin genes (*tcdA* and *tcdB*) in *C. difficile* isolates was studied by PCR amplification. *tcdA*+/*tcdB*+ pattern constituted a major subtype (86%); however, there exists a gradually increasing trend of *C. difficile* isolates in Korea exhibiting a *tcdA*−/*tcdB*+ subtype. A study comparing the expression pattern of *C. difficile tcdA/B* toxins in isolates from CDI patients reported that *tcdA*−/*tcdB*+ subtype constituted 7% of subtypes detected in 2002 but rose to 27.0% by 2005 [13]. Toxin B is 1,000-fold more toxic than toxin A [14]. *tcdA*−/*tcdB*+ subtype is the result of a mutation in the repeat sequence of *tcdA* gene [2]. Hyper-virulent strains related to severe CDI are called as restriction endonuclease-analysis group B1/North American pulsed-field gel electrophoresis type 1 (NAP1)/ribotype 027 (B1/NAP1/

ribotype 027). A change in the sequence of the *tcdC* repressor gene results in the loss of its inhibitory function against the transcription of *tcdA* and *tcdB* in the virulent B1/NAP1/ribotype 027 strain. Therefore, this strain produces 16-fold higher concentrations of toxin A and 23-fold higher concentrations of toxin B, which results in the resistance to fluoroquinolone series of antibiotics [15]. Furthermore, this strain produces a third toxin called binary toxin (actin-specific ADP-ribosyltransferase), which exhibits synergistic effects with toxins A and B, thereby leading to more severe CDIs [16]. The occurrence of recurrent stomach PMC in patients infected with strain BI/NAP1/ribotype 027 is suggestive of the increase in its repetition rate [15]. In this study, *tcdB* was detected in all isolates. The toxigenic properties of *C. difficile* strains isolated from patients with CDAD in Korea have continued to increase, as previously reported [13]. Therefore, it is essential to examine toxin B (encoded by *tcdB*) along with toxin A (encoded by *tcdA*) for the accurate detection of *C. difficile* and diagnosis of CDI.

C. difficile is among the major bacteria associated with HAIs and the infection is known to be transmitted by spores in most hospitals [12]. All *C. difficile* isolates tested in this study showed spore formation; therefore, care must be taken to prevent infections in hospitals. CDI is classified as moderate or severe with complications, and metronidazole and vancomycin have been used as primary drugs for CDI treatment [14]. Results of the antibiotic susceptibility test showed that all isolates were susceptible to vancomycin and metronidazole. Rep-PCR is a very powerful tool used for the determination of bacterial lineages [17]. Here, we used rep-PCR to characterize *C. difficile* lineages based on the correlation between the information from patients and *C. difficile* isolates. We observed that the differences in the banding patterns in rep-PCR were associated with different diseases in patients. Two *C. difficile* isolates (number 6 and 7) exhibited > 99% similarity in their banding patterns. Although these two isolates were obtained from different patients, these two patients were hospitalized in the same ward and at the same time, indicating that these genetically identical strains may have originated from HAI. Conversely, most tested isolates showed distinguishable banding patterns, indicating that automated rep-PCR may be considered as an effective subtyping tool for tracking the source of infections (e.g., hospital- or community-acquired CDAD). In the case of CDI, prevention is more important than treatment. Development of vaccines against *C. difficile* was previously attempted [18]; however, no effective vaccine has been developed yet.

In conclusion, our study on the characterization of *C. difficile* provided useful information for developing strategies to prevent CDI, especially hospital- and community-acquired CDAD. These results contribute to the accurate determination of suitable antibiotic treatment and drug development against *C. difficile*.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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