Emergence of *Clostridium difficile* Ribotype 027 in Korea

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Background: *Clostridium difficile* infection (CDI) has markedly risen and is associated with hypervirulent ribotype 027 outbreaks in North America and Europe since 2003. The aims of this study were to determine the prevalence of ribotype 027 among *C. difficile* isolates in Korea, to characterize the ribotype 027 isolates, and to determine the clinical severity of CDI in patients infected with these isolates.

Methods: A total of 1,251 isolates of *C. difficile* recovered from stool specimens of suspected CDI patients at two tertiary-care hospitals and one commercial laboratory between 2002 and 2009. Genes for toxin A (*tcdA*), toxin B (*tcdB*), and binary toxin (*cdtA* and *cdtB*) were detected by PCR. Mutation in the *tcdC* gene was detected by sequencing after PCR amplification. For molecular genotyping, we performed PCR-ribotyping, pulsed-field gel electrophoresis (PFGE), and multilocus variable-number tandem-repeat analysis (MLVA). Minimum inhibitory concentrations of moxifloxacin were determined using Etest strips (AB bioMérieux, Sweden).

Results: We identified 7 isolates as ribotype 027. These isolates had the same *tcdC* mutation as the epidemic strain, and 6 of them were resistant to moxifloxacin. The isolates were categorized into 3 different PFGE types and 7 different MLVA types. All the 7 cases had occurred sporadically.

Conclusions: *C. difficile* ribotype 027 is uncommon, but it has emerged in Korea. The spread of this ribotype should be closely monitored in order to avoid an outbreak of CDI in Korea.

Key Words: *Clostridium difficile*, PCR-ribotype 027, Pulsed-field gel electrophoresis, Multilocus variable-number tandem-repeat analysis

INTRODUCTION

Severe *Clostridium difficile* infections (CDIs) associated with an emerging epidemic strain, PCR-ribotype 027, have increased in frequency and severity in the U.S., Canada, and the European countries since 2003 [1]. However, reports of ribotype 027 infections in East Asian countries remain rare [2]. One case each was reported from Japan [3] and Korea [4] in 2007 and 2009, respectively. A one-year surveillance study between 2007 and 2008 in Shanghai, China failed to detect any cases, although one case was reported in Hong Kong in 2009 [2]. These results may indicate the onset of spreading ribotype 027 infection in Asian countries.

Ribotype 027 strain had toxin A, toxin B and binary toxin with deletion of toxin regulator gene (*tdcC*) [1]. According to a previous study, the use of moxifloxacin is associated with ribotype 027 outbreaks, and the isolates obtained in that study were resistant to levofloxacin and moxifloxacin [5]. A Korean isolate was resistant, whereas 1 Japanese isolate was susceptible to moxifloxacin [3, 4]; this indicated a need for further testing of Korean isolates.

The aims of this study were to determine the prevalence of ribotype 027 among the stored *C. difficile* isolates, to characterize the ribotype 027 isolates, and to evaluate the clinical severity of CDI in patients infected with these strains.
**METHODS**

1. **Bacterial isolates and antimicrobial susceptibility testing**
   A total of 1,251 *C. difficile* isolates were recovered from stool specimens of suspected CDI patients at two tertiary-care hospitals and one commercial laboratory between 2002 and 2009. The commercial laboratory processed the stool specimens collected from hospitals and clinics without in-house anaerobic microbiological testing facilities. *C. difficile* selective agar (CDSA; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was used to isolate *C. difficile* under anaerobic incubation. The isolates were identified using conventional tests and the ATB 32A system (bioMerieux, Marcy l’Etoile, France). Minimum inhibitory concentrations (MICs) of moxifloxacin for ribotype 027 isolates were determined using Etest strips (AB bioMérieux, Solna, Sweden).

2. **Clinical characteristics of patients**
   We reviewed the patients’ medical records, and according to the criteria proposed by Zar et al. [6], patients with ≥2 points were considered to have severe CDI. One point was given for each of the following criteria: age >60 yr, body temperature >38.3 °C, serum albumin <2.5 mg/dL, and peripheral white blood cell count >15,000/μL. Two points were given if there was endoscopic evidence of pseudomembranous colitis (PMC) or if the patient was admitted to the intensive care unit.

3. **PCR assay for toxin genes**
   *C. difficile* toxin genes were detected by performing PCR as described in previous studies [7, 8]. Primer sequences used in this study are listed in Table 1. *C. difficile* VPI 10463 (A+ B+, CDT−), 3608/03 (A− B−, CDT−), SE844 (A+ B+, CDT+), and 1470 (A− B+, CDT−) were used as controls for the PCR assays. The obtained amplicons were commercially sequenced (Macrogen, Seoul, Korea). The deduced amino acid sequences were compared to those of the strain VPI 10463.

4. **PCR-ribotyping**
   PCR-ribotyping was performed according to a previously described method [9] with minor modification. We added 10 μL of crude nucleic acid as template to a 50-μL PCR mixture containing 10 mM Tris-HCl (pH 8.3) and 50 mM KCl (GeneAmp 1×PCR Buffer II; Applied Biosystems, Foster City, CA, USA), 4.0 mM MgCl₂, 0.4 mM dNTP, 1.5 U Taq DNA polymerase (AmpliTaq Gold, Applied Biosystems), and 0.4 μM primers (CD1 and CD1445).

5. **Pulsed-field gel electrophoresis and multilocus variable-number tandem-repeat analysis typing**
   Pulsed-field gel electrophoresis (PFGE) analysis was performed by modified method of Alonso et al. [10]. In brief, we prepared plugs of chromosomal DNA from fresh colonies cultured for 24 hr and treated them with high concentration of proteinase K (75 U/mL). Thiourea was added to the gel and running buffer used for PFGE [11].
   Multilocus variable-number tandem-repeat analysis (MLVA) was performed by PCR amplification and sequence analysis of 7 selected *C. difficile* repeat (CDR) loci: CDR4, CDR5, CDR9, CDR48, CDR49, CDR59, and CDR60. The primer sets used are listed in Table 1 [12]. The copy numbers of each of the 7 CDR loci were concatenated to generate an MLVA type for each isolate.

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| Table 1. Primers used in this study |
|-------------------------------------|
| **Test** | **Target** | **Primer** | **Oligonucleotide sequence (5’→3’)** | **Reference** |
|-------------------------------------|
| Toxin genes | tcdA | NK9 | CCACCAGCTGCGACCATA | [7] |
| | rep | NK11 | TGAGCTCAAAAGAATCTAAAATGGTAAC | |
| | tcdB | NK104 | GTCTGACATGAAAGTCCAGTATTACGC | |
| | NK105 | CATCTGCTTCTTAGGGTCAGCT | |
| | cdtA | cdtA pos | TGAACCTGAAAAGGAGT4GT | |
| | cdtA rev | AGATATTTACGTGACCATTTTG | |
| | cdtB | cdtB pos | GGTGAGTGAATCCGGACGAAC | |
| | cdtB rev | AACGGACTCTTGCGTCAGTC | |
| PCR | 16S-23S | CD1 | GCCCCTTTGTTAGCCTGACC | [9] |
| | ribotyping | rRNA | CD1445 | CGGGGTGAAAGCTGAAACAGG |
| | tcdC | tcdC | Pal15 | TCTCTACGCTATCCCTGTT |
| | mutation | Pal16 | AAAAAGGAGGAGAATGTAT | |
| MLVA | CDR4F | CDR4F | ATTAATCATACTACAAACACGA | [12] |
| | CDR4R | CDR4R | TAAACAAATGAAATCTAAAGAAGG | |
| | CDR5F | CDR5F | AATTTATAGAAGCTTTTCTCAT | |
| | CDR5R | CDR5R | AGCCATTTTACAGCTCCCAT | |
| | CDR9F | CDR9F | GGTGGAGTGAATCCGGACGAAC | |
| | CDR9R | CDR9R | GGTGGAGTGAATCCGGACGAAC | |
| | CDR48F | CDR48F | AGGAGGTTTATGAGAAGTACAGG | |
| | CDR48R | CDR48R | ATGTATTTTACATCCGGACGAAC | |
| | CDR49F | CDR49F | AACCATATTAGGAACTTGATC | |
| | CDR49R | CDR49R | GATTATATTATTTTACATCCGGACGAAC | |
| | CDR59F | CDR59F | GTAGAAGGCGAAAATAGAAG | |
| | CDR59R | CDR59R | CCTCTGCTTGCTGTAGAATA | |
| | CDR60F | CDR60F | GGTGCGACAGCTGTGCTGCTG | |
| | CDR60R | CDR60R | AAGGGATGCTTATCCCTGATA | |

Abbreviations: MLVA, multilocus variable-number tandem-repeat analysis; CDR, *Clos tridium difficile* repeat.
RESULTS

Of the 1,251 C. difficile isolates, 47 (3.8%) were PCR positive for the toxin A, toxin B, and binary toxin genes, and 7 (0.6%) were identified as ribotype 027 by PCR-ribotyping. The first isolate to be identified as ribotype 027 was from a sample obtained in 2006, and the prevalence of ribotype 027 was 0.6% in 2006 and 2007, 0.9% in 2008, and 1.0% in 2009 (Table 2).

All the ribotype 027 isolates had an 18-bp deletion from position 330 to 347 and a 1-bp deletion at position 117 in the tcdC gene sequence. We observed identical PFGE patterns for 5 isolates (isolates 1, 4, 5, 6, and 7). However, the patterns for isolates 2 and 3 differed from those of the other isolates in 3 and 2 bands, respectively (Table 3, Fig. 1).

All the 7 isolates had different MLVA types. However, all the isolates had the same number of variable number tandem repeats (VNTRs) at the CDR5 and CDR59 loci (Table 3).

The MIC of moxifloxacin was >32 μg/mL for 6 of the 7 isolates and 0.5 μg/mL for the remaining 1 isolate (isolate 2).

Table 2. Prevalence of binary toxin-producing Clostridium difficile and PCR-ribotype 027

| Year | N of isolates | Total | Binary toxin (+) | Ribotype 027 |
|------|---------------|-------|------------------|--------------|
|      | S hospital    | Y hospital | Commercial Lab. |               |
| 2002 | 179           | ND     | ND               | 179          |
| 2003 | 107           | ND     | ND               | 107          |
| 2004 | 53            | ND     | ND               | 53           |
| 2005 | 40            | 2      | ND               | 42           |
| 2006 | 155           | 19     | ND               | 174          |
| 2007 | 145           | 13     | ND               | 158          |
| 2008 | 162           | 58     | 109              | 329          |
| 2009 | 98            | 23     | 88               | 209          |
| (Jan–Jun) | | | | |
| Total | 939 | 115 | 197 | 1,251 |

Abbreviation: ND, not done.

Table 3. Molecular and phenotypic characteristics of the Clostridium difficile PCR-ribotype 027

| No. of Isolate | Year of Isolation | Hospital* | PFGE type | mlva (N of VNTRs for each CDR locus) | MIC of moxifloxacin (μg/mL) |
|----------------|-------------------|-----------|-----------|-------------------------------------|----------------------------|
| 1              | 2006              | Y         | I         | CD R 4 | CD R 5 | CD R 9 | CD R 48 | CD R 49 | CD R 59 | CD R 60 | >32 |
| 2              | 2007              | S         | III       | 3       | 14     | 9      | 12      | 1       | 12      | >32 |
| 3              | 2008              | K         | II        | 3       | 14     | 9      | 13      | 1       | 9       | >32 |
| 4              | 2008              | K         | I         | 3       | 15     | 9      | 12      | 1       | 10      | >32 |
| 5              | 2008              | G         | I         | 3       | 14     | 9      | 12      | 1       | 10      | >32 |
| 6              | 2009              | Y         | I         | 3       | 14     | 9      | 13      | 1       | 10      | >32 |
| 7              | 2009              | S         | I         | 3       | 16     | 10     | 12      | 1       | 9       | >32 |

*Two hospitals, hospitals K and G, sent the samples to a commercial laboratory, and the samples were redirected to the S hospital in Seoul where the test was performed.

Fig. 1. Pulsed-field gel electrophoresis (PFGE) patterns of Smal-digested genomic DNA obtained from the ribotype 027 isolates. Lanes 1 and 9 were loaded with a 50-kb ladder (molecular marker). The PFGE patterns for isolates 2 and 3 differed in 5 bands. The remaining 5 isolates had identical patterns, and their pattern differed from that of isolates 2 and 3 in 3 and 2 bands, respectively.
| No. of patient | N of days from admission to isolation | Duration of admission | Age/ Gender | Underlying disease | Previous therapy | Diarrhea times/day | WBC (x10^9/L) | Serum albumin (mg/dL) | Colonoscopy/ Biopsy | Treatments | Outcome |
|----------------|--------------------------------------|----------------------|-------------|-------------------|-----------------|-------------------|----------------|------------------------|-----------------|------------|---------|
| 1              | 1 day (had previous admission history) | 26 May-1 Jun 2006    | 88 yr/F     | Pulmonary tuberculosis | HERZ for 2 mo   | 3-4               | 4,900          | 3.3                    | Suspected ulcerative colitis, infectious colitis/Within normal limit | Ceftriaxone          | Improved |
| 2              | 5 days                               | 30 Mar-24 Apr 2007   | 25 yr/F     | CRF, AV fistular infection | Cefobactam/ vancomycin for 20 days | 5-10            | 17,180         | 2.1                   | Suspected Pseudomembranous colitis/Chronic nonspecific inflammation | Metronidazole          | Improved |
| 3              | 16 days                              | 30 Jun-22 Jul 2008   | 73 yr/F     | CLL, pulmonary tuberculosis | HERZ for 3 mo, Fludarabine + cyclophosphamide for 3 days, gancyclovir for 2 wk, cefepime + metronidazole for 2 wk | Over 10         | 730             | 3.0                   | NT              | Cefepime, metronidazole | Improved |
| 4              | 86 days                              | 4 Aug-18 Nov 2008    | 53 yr/M     | Wound infection (MRSA) | Vancomycin for 4 mo, ciprofloxacin for 1 wk | 5-10            | 7,200          | 3.0                   | NT              | Metronidazole, vancomycin | Recurred |
| 5              | 14 days                              | 18 Nov-11 Dec 2008   | 40 days/M   | Acute gastroenteritis | Data not available | 5-10            | 7,790          | 3.8                   | NT              | Amikacin, piperacillin - tazobactam | Not applicable |
| 6              | 2 days                               | 16 Mar-20 Mar 2009   | 62 yr/F     | Tuberculous lymphadenitis | HERZ for 3 mo | 5-10            | 7,600          | 4.1                   | Infectious colitis/ Increased number of plasma cells in the lamina propria | Ciprofloxacin           | Improved |
| 7              | 28 days                              | 19 Mar-28 Apr 2009   | 82 yr/F     | Pulmonary tuberculosis | HERZ for 2 mo   | 5-10            | 9,630          | 2.9                   | Pseudomembranous colitis/ Acute colitis, active | Metronidazole, vancomycin | Recurred |

Abbreviations: WBC, white blood cell; HERZ, isoniazid, ethambutol, rifampin, pyrazamide; mo, month; CRF, chronic renal failure; AV, arteriovenous; NT, not tested; MRSA, methicillin-resistant Staphylococcus aureus; HER, isoniazid, ethambutol, rifampin.
patients had travelled overseas. Symptoms and their severity varied among the patients, ranging from mild colitis to severe PMC. Except for patient 2, none of the patients showed high fever (>38.0°C), increased leukocyte count (>15,000/μL), increased serum creatinine (>2.0 mg/dL), or decreased serum albumin (<2.5 mg/dL). Patient 2 showed high leukocyte count and serum creatinine level due to underlying chronic renal failure and an arteriovenous fistula infection.

Two (patient nos. 2 and 7) cases were diagnosed with suspected PMC and PMC by colonoscopic biopsy and considered probably severe (patient no. 2) or severe (patient no. 7) according to the criteria proposed by Zar FA et al. [6].

Metronidazole was administered to 4 patients (patient nos. 2, 3, 4, and 7) to treat CDI. Because of poor response, for patients 4 and 7, the regimen was changed to vancomycin, but the infection recurred. Two patients (patient nos. 1 and 6) recovered without CDI treatment.

**DISCUSSION**

Although the first case of ribotype 027 infection in Korea was reported in 2009 [4], we found that ribotype 027 was present in Korea as early as 2006. Since 2006, the prevalence of ribotype 027 isolates has been 0.8% (7/870). Except for patient no. 6, all the patients had most likely acquired the infection in a hospital. We recovered these isolates from 7 patients who had visited 4 different hospitals located in Seoul and Gyeonggi province; this indicated an early stage of dissemination to Korean hospitals. Two of these hospitals sent the samples to a commercial laboratory for culturing *C. difficile*, and the samples were redirected to the S hospital in Seoul, Korea, and Gwanghee Byun (Kyunghee University, Seoul, Korea), Kwangwoo Kim (Yonsei University Health System, Seoul, Korea), and Myungsook Kim (Yonsei University Health System, Seoul, Korea) for laboratory assistance.

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