The changes of PCR ribotype and antimicrobial resistance of *Clostridium difficile* in a tertiary care hospital over 10 years

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The aims of this study were to investigate any change in PCR ribotypes and to determine the antimicrobial resistance of common PCR ribotypes over a 10-year period in a tertiary care hospital. We conducted PCR ribotyping, antimicrobial susceptibility testing and DNA gyrase sequencing to identify changes in 1407 *Clostridium difficile* non-duplicated isolates obtained between 2000 and 2009. A total of 74 different ribotypes were found. The most prevalent ribotype was ribotype 001 (26.1 %). The prevalence of ribotype 017 was 17 % and that of ribotype 014/020 was 9.6 %. Ribotyping showed that the prevalence of ribotype 001 decreased and the prevalence of ribotypes 017, 014/020 and 018 increased over the 10 years. Antimicrobial resistance rates in prevalent ribotypes were: clindamycin, 81 %; cefotetan, 19 %; moxifloxacin, 42 %; imipenem, 8 %; ciprofloxacin, 100 % and erythromycin, 80 %. Ribotype 018 showed greater antimicrobial resistance than other ribotypes. All ribotype 018 strains showing moxifloxacin resistance had a substitution of a *gyrA* coding amino acid (Thr82 to Ile). This study will help the understanding of PCR ribotype trends and antimicrobial resistance of *C. difficile* in Korea.

**INTRODUCTION**

*Clostridium difficile* infection (CDI) can cause mild to severe clinical problems such as pseudomembranous colitis, antibiotic-associated diarrhoea and colitis (Lyerly et al., 1988). Toxin A (TcdA) and toxin B (TcdB) of *C. difficile* are known to play major roles in the pathogenesis of CDI. Some strains yield a binary toxin referred to as *C. difficile* toxin (CDT), but its exact role in CDI is unclear (Barbut et al., 2005). The incidence of CDIs has been increasing, and morbidity and mortality rates associated with hypervirulent strains known as North American PFGE type 1, restriction endonuclease analysis group BI or PCR ribotype 027 (NAP1/BI/027) have increased (O’Donoghue & Kyne, 2011). In Europe, an epidemiological study of CDI across 34 European countries during November 2008 identified 65 different *C. difficile* PCR ribotypes, among which ribotypes 014/020 (16 %), 001 (9 %) and 078 (8 %) were the most prevalent, while the prevalence of ribotype 027 was 5 % (Bauer et al., 2011). In North America, a study of the distribution of *C. difficile* strains showed NAP1/BI/027 was the most frequently isolated strain and accounted for 36 % of isolates from 2005 to 2007 (Cheknis et al., 2009). While there have been few reports regarding the epidemiology of CDI in Asia, Tae et al. (2009) reported on the first case of antibiotic-associated colitis by ribotype 027 in Korea. The predominant ribotype was reported to be ribotype 017 (25.7 %) for Korea in 2006–2008 (Kim et al., 2010). However in China, a collection of 75 clinical isolates from Shanghai in 2008 showed that 33 % were ribotype 017 strains, but no ribotype 027 strains were present (Huang et al., 2009a). The predominant ribotype in Japan was ribotype f in 2004 (18/28 cases, 64 %), which corresponds with ribotype 001 (Sawabe et al., 2007).

The prevalent ribotype of *C. difficile* differs geographically. Resistance to antimicrobial agents in *C. difficile* may have played a part in its selection in the hospital environment. The aims of this study were to investigate any change in PCR ribotypes and to determine the antimicrobial resistance of common PCR ribotypes over a 10-year period in a tertiary care hospital in Korea.

**METHODS**

**Bacterial strains.** A total of 1407 unduplicated clinical isolates of *C. difficile* recovered from patients whose liquid stool specimens were

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**Abbreviations:** CDI, *Clostridium difficile* infection; CDT, *C. difficile* toxin; TcdA, toxin A of *C. difficile*; TcdB, toxin B of *C. difficile*; QRDR, quinolone resistance-determining region.
C. difficile culture and toxin gene analysis by PCR. Stool specimens were cultured anaerobically on C. difficile selective agar (Becton Dickinson) at 37 °C in an anaerobic chamber (Forma Scientific) for 48 h. Before February 2006, home-made cycloserine-cefoxitin-fructose agar was used. Species identification was performed on the basis of typical morphology of colonies on agar plates as well as characteristic odour and ATB 32A systems results (bioMérieux). C. difficile toxin genes (tcdA, tcdB, cdaA and cdbB) were detected by PCR as described previously (Kato et al., 1998; Stubbs et al., 2000). The primer pairs used were NK9/NK11 for the repetitive domain of tcdA, NK104/NK105 for tcdB, cdaA pos/cdbB rev for cdaA, and cdbB pos/cdbB rev for cdbB.

PCR ribotyping. PCR ribotyping was performed as previously described using the primers 5′-CTGGGTTGAAGTGTTAAACAG-G-3′ (nt 1445–1466 of the 16S rRNA gene) and 5′-GCGCCCTTGT-AGCTTGACC-3′ (nt 20–1 of the 23S rRNA gene) (Stubbs et al., 1999). Comparison of PCR ribotyping patterns was performed visually with known standards. Strains with ribotype patterns that differed by at least one band were assigned to different types. Ribotype groups were designated by upper- and lower-case letters combined with a number.

Antimicrobial susceptibility testing. Antimicrobial susceptibility tests were performed with 120 randomly selected C. difficile strains of four common ribotypes identified during the study period using the agar dilution method on Brucella blood agar, according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2012). MIC breakpoints of clindamycin (Korea Upjohn), cefotetan (Daichi Pharmaceutical), moxifloxacin (Bayer Korea) and imipenem (ChoongWae) were referenced using the CLSI guidelines (CLSI, 2012). If no standard MIC breakpoint had been defined, such as for ciprofloxacin (Bayer Korea) and erythromycin (Sigma), >4 and ≥8 mg l⁻¹, respectively, were used (CLSI, 2013).

DNA gyrase A (gyrA) and gyrase B (gyrB) mutation assay. Molecular analysis of the gyrA and gyrB genomic regions included the amplification and sequencing of the quinolone resistance-determining region (QRDR) of gyrA and gyrB from 120 C. difficile isolates using a procedure described previously (Drudy et al., 2006).

RESULTS

Toxin analysis
A total of 864 (61.4 %) TcdA-positive and TcdB-positive (A⁺B⁺) strains and 239 (17 %) TcdA-negative and TcdB-positive (A⁻B⁺) strains were identified. The overall prevalence of binary toxin-positive (CDT⁺) strains among the strains was 3.1 %; the highest prevalence was 5.5 % in 2007 and the lowest was 0 % in 2003. All CDT⁺ strains were also A⁺B⁺. A total of 304 (21.6 %) A⁺B⁻ CDT⁻ strains were identified. The 10-year trend for CDT patterns is described in Fig. 1.

PCR ribotypes
Of the 1407 isolates, we identified 74 different ribotypes. All A⁻B⁺ strains showed the same banding pattern, which was identical to the pattern of C. difficile 1470 (ribotype 017) strain. The five most common ribotypes (and corresponding percentage prevalence) were ribotype AB1 (ribotype 001, 26.1 %), ribotype aB (ribotype 017, 17 %), ribotype AB2 (ribotype 014/020, 9.6 %), ribotype AB3 (5.6 %) and ribotype AB17 (ribotype 018, 4.1 %). Among them, ribotypes 001, 014/020, 017 and 018 constituted more than 10 % of the isolates for each year. Changes in prevalence are shown in Fig. 2.

Antimicrobial resistances
The antimicrobial resistance rates of 120 C. difficile isolates were as follows: clindamycin, 81 %; cefotetan, 19 %; moxifloxacin, 42 %; imipenem, 8 %; ciprofloxacin, 100 %; erythromycin, 80 %. Antimicrobial resistance according to each ribotype is shown in Table 1. Ribotypes 001, 017 and 018 showed 100 % resistance to clindamycin and erythromycin. Ribotypes 017 and 018 showed 85 and 100 % resistance, respectively, to moxifloxacin. Ribotype 018 had the highest overall resistance, while ribotype 014/020 had the lowest antimicrobial resistance compared with other ribotypes.

DNA gyrase A (gyrA) and DNA gyrase B (gyrB) mutation assay
All isolates with moxifloxacin resistance had a mutation resulting in an amino acid substitution in gyrA or gyrB. Among 50 moxifloxacin-resistant isolates, 72 % (36/50) had a substitution in gyrA, 14 % (7/50) had mutations in both gyrA and gyrB, and 14 % (7/50) had a single mutation in gyrB. In particular, there were mutations according to each ribotype from 120 tested sequencing results (Table 2).
DISCUSSION

*C. difficile* ribotypes showed different prevalence rates during the study period in Korea. Common ribotypes were 001 (26.1 %), 017 (17 %), 014/020 (9.6 %) and 018 (4.1 %), which constituted more than 10 % of the isolates for each year. In particular, ribotype 001 steeply decreased from the year 2000 and ribotype 017 strains were the most prevalent ribotype from 2004 to 2008. Ribotype 018 has been identified since 2006 and was the most common type in 2009. One strain of ribotype 027 identified in 2007 was susceptible to moxifloxacin, so this strain was considered to be an historic ribotype 027 strain, unlike hypervirulent *C. difficile* 027/BI/NAP1.

It was thought that the antimicrobial resistance of *C. difficile* strains may be a factor that contributes to the persistence and dissemination of the strain in the hospital environment (McDonald *et al.*, 2005; Drudy *et al.*, 2006). We assumed that the prevalent ribotypes would show high antimicrobial resistance rates. We noted that the most prevalent ribotypes were, in order, ribotype 001 and 017, and ribotype 018. Compared with ribotype 001, ribotypes 017 and 018 had high MICs for moxifloxacin and imipenem. In our hospital, moxifloxacin and imipenem have been used since 2003 and the introduction of these drugs would be related to the shift of ribotype.

Ribotype 018 strain demonstrated the highest degree of antimicrobial resistance while ribotype 014/020 showed lower resistance when compared with other ribotypes. Despite this, ribotype 014/020 was identified commonly throughout the study period and it continues to be a dominant ribotype worldwide (Bauer *et al.*, 2011). The antimicrobial resistance rates were significantly different

**Table 1.** MICs of six antimicrobial agents for Korean *C. difficile* isolates according to PCR ribotype

<table>
<thead>
<tr>
<th>Ribotype 001 (A^+B^+CDT^-) (n=52)</th>
<th>Ribotype 014/020 (A^+B^+CDT^-) (n=26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antimicrobial</td>
<td>MIC (µg ml^-1)</td>
</tr>
<tr>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Cefotetan</td>
<td>16–32</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>4–8</td>
</tr>
<tr>
<td>Imipenem</td>
<td>2–8</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>32–128</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>≥128</td>
</tr>
<tr>
<td>Ribotype 018 (A^+B^+CDT^-) (n=8)</td>
<td>Ribotype 017 (A^-B^-CDT^+) (n=34)</td>
</tr>
<tr>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Antimicrobial</td>
<td>MIC (µg ml^-1)</td>
</tr>
<tr>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Cefotetan</td>
<td>64</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>16</td>
</tr>
<tr>
<td>Imipenem</td>
<td>8–16</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>64–128</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>&gt;128</td>
</tr>
</tbody>
</table>

S, susceptible; I, intermediate; R, resistant.

Fig. 2. Prevalent PCR ribotype changes of *C. difficile* over 10 years. ●, Ribotype 001; ■, ribotype 014/020; ●, ribotype 018; X, ribotype 017.

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between each ribotype. Also, no definite change in antimicrobial resistance rates of prevalent ribotype strains was identified according to each year in our study. Thus, other factors should be considered in addition to antimicrobial resistance in relation to the mechanisms of ribotype selection in the hospital environment.

Recent studies have reported that rates of resistance to moxifloxacin in *C. difficile* have dramatically increased to 37.5% (Barbut *et al.*, 2007), over 80% (Bourgault *et al.*, 2006) and 46.4% (Huang *et al.*, 2009c) compared with a previous report from France (7% reported) in 1991 and 1997 (Dridi *et al.*, 2002) and another from Germany (12% reported) between 1986 and 2001 (Ackermann *et al.*, 2003). However, the present study did not identify definite annual changes in antimicrobial resistance rates of prevalent ribotype strains. The increased rate of antimicrobial resistance seen in these studies would appear to be related to changes in the prevalent ribotypes.

Generally, fluoroquinolone resistance is acquired in two ways, either by target enzyme alterations or by reduced intracellular concentrations (Huang *et al.*, 2009b). However, some studies determined that the MICs of fluoroquinolones were not significantly affected by the addition of efflux pump inhibitors (Drudy *et al.*, 2006; Huang *et al.*, 2009c). These studies identified alterations in the QRDRs of both DNA gyrase subunits, *gyrA* and *gyrB*, which are known to be involved in *C. difficile* quinolone resistance (Ackermann *et al.*, 2001; Drudy *et al.*, 2006). The ribotype 018 was predominant in 2007 and 2008 in Italy, and the majority of the fluoroquinolone-resistant isolates seen in that country belonged to ribotype 018 or 126 (Spigaglia *et al.*, 2010). In this study, all resistant ribotype 001 and 018 strains had a substitution of a *gyrA* coding amino acid, Thr82 to Val and Thr82 to Ile, respectively. DNA gyrase subunit *gyrA* was also identified as the main target of a single nucleotide substitution in a European surveillance study (Spigaglia *et al.*, 2008) with 83% of moxifloxacin-resistant *C. difficile* isolates having a substitution in *gyrA* (Thr82 to Ile). There was no novel mutation found in *gyrA* or *gyrB* in this study. High-level resistance to fluoroquinolones due to an Asp426 to Val amino acid substitution in *gyrB* was reported in the A B + C. *difficile* strains (Drudy *et al.*, 2006; Spigaglia *et al.*, 2008). However, the same substitution was found in A B + strains (ribotype 014/020) showing low-level resistance in this study. Also, in a study in Shanghai, A B + strains carried the same mutation, but it could not be determined whether this mutation was associated with high-level resistance because of a combined mutation in *gyrA* (Huang *et al.*, 2009c). In particular, ribotype 017 strains with double mutations of *gyrA* and *gyrB* showed higher MICs than strains with a *gyrA* single mutation in our survey. Based on these analyses, we hypothesize that double alterations of the QRDR region might cause a conformational change that enhances moxifloxacin resistance in ribotype 017 strains as reported previously (Heddle & Maxwell, 2002). However, additional studies are necessary to investigate the association between the specific substitutions and specific ribotypes with high-level antimicrobial resistance.

Our study was performed at a large tertiary healthcare centre, and it is the first to investigate 10-year trends in the changes of PCR ribotypes, antimicrobial resistance of common ribotypes and amino acid alteration of QRDR of *C. difficile* isolates in South Korea. The findings in this report are subject to at least two considerations. Firstly, these cases were mainly hospital acquired. Most strains of *C. difficile* were isolated from patients in hospital wards. Strains isolated from patients in outpatient departments and emergency rooms were 5.8% of the total isolates in 2002–2004 and 4.0% in 2008–2009, respectively. Secondly, there were no detectable outbreaks of specific ribotypes of *C. difficile* in our hospital during the study period. We have routinely performed *C. difficile* culture since 1985. However, because CDI had received little attention before the emergence of the hypervirulent *C. difficile* 027/BI/NAP1 strain, small outbreaks of CDI could have been ignored.

Although hypervirulent *C. difficile* 027/BI/NAP1 is not common in Korea, outbreaks of other hypervirulent types might be possible. Therefore the organization of a nationwide surveillance system should be considered for efficient control of CDI.

In conclusion, *C. difficile* ribotyping showed that there was a shift in which the prevalence of ribotype 001 decreased and

<table>
<thead>
<tr>
<th>Ribotype</th>
<th>DNA gyrase A</th>
<th>No. of isolates</th>
<th>DNA gyrase B</th>
<th>No. of isolates</th>
<th>MIC of moxifloxacin (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribotype 001 (n=52)</td>
<td>Thr82 to Val</td>
<td>52 (100%)</td>
<td>Thr82 to Ile</td>
<td>1 (3.9%)</td>
<td>4–8</td>
</tr>
<tr>
<td>Ribotype 014/020 (n=26)</td>
<td>Thr82 to Val</td>
<td>16 (6.2%)</td>
<td>Asp426 to Asn</td>
<td>2 (7.7%)</td>
<td>16</td>
</tr>
<tr>
<td>Ribotype 014/020 (n=26)</td>
<td>Thr82 to Ile</td>
<td>8 (3.2%)</td>
<td>Asp426 to Val</td>
<td>5 (19.2%)</td>
<td>16</td>
</tr>
<tr>
<td>Ribotype 017 (n=8)</td>
<td>Thr82 to Ile</td>
<td>22 (64.7%)</td>
<td>Asp426 to Asn</td>
<td>7 (20.6%)</td>
<td>16–128</td>
</tr>
<tr>
<td>Ribotype 018 (n=8)</td>
<td>Thr82 to Ile</td>
<td>7 (20.6%)</td>
<td>Asp426 to Asn</td>
<td>7 (20.6%)</td>
<td>128–&gt;128*</td>
</tr>
</tbody>
</table>

*In three cases, the MIC was higher than 128 µg ml⁻¹.*
the prevalence of ribotypes 017, 014/020 and 018 increased over 10 years. Differences of antimicrobial resistance were noted in different ribotypes. This study will be helpful to aid the understanding of the PCR ribotype trends and antimicrobial resistance of \textit{C. difficile} in Korea.

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