Predominance of PCR-ribotypes, 018 (smz) and 369 (trf) of Clostridium difficile in Japan: a potential relationship with other global circulating strains?

Mitsutoshi Senoh,1 Haru Kato,1 Tadashi Fukuda,1 Akiko Niikawa,2 Yoshiko Hori,3 Hideharu Hagiya,4 Yoichiro Ito,5 Hiroshi Miki,6 Yoshifumi Abe,6 Kiyoshi Furuta,7 Hideki Takeuchi,7 Hirokazu Tajima,8 Harumi Tominaga,9 Hideyuki Satomura,10 Hideaki Kato,11 Sayuri Morita,11 Ai Tanada,11 Toshinori Hara,12 Miki Kawada,13 Yuka Sato,13 Masahiko Takahashi,14 Akiko Higuchi,14 Tomoko Nakajima,15 Yukiko Wakamatsu,15 Masahiro Toyokawa,16 Akiko Ueda,16 Paul Roberts,17 Fabio Miyajima18 and Keigo Shibayama1

1Department of Bacteriology II, National Institute of Infectious Diseases, Tokyo, Japan
2Ishikawa Prefectural Central Hospital, Ishikawa Japan
3Okayama Saiseikai General Hospital, Okayama, Japan
4Tsuyma Chuo Hospital, Okayama, Japan
5Japanese Red Cross Gifu Hospital, Gifu, Japan
6NHO Sendai Medical Center, Miyagi, Japan
7NHO Matsumoto Medical Center, Nagano, Japan
8Aichi Cancer Center Hospital, Aichi, Japan
9NHO Kure Medical Center, Hiroshima, Japan
10Chiba Cancer Center, Chiba, Japan
11Toyokawa City Hospital, Aichi, Japan
12Hiroshima University Hospital, Hiroshima, Japan
13Saitama City Hospital, Saitama, Japan
14NHO Tokyo Medical Center, Tokyo, Japan
15Tsuruoka Municipal Shonai Hospital, Yamagata, Japan
16Osaka University Hospital, Osaka, Japan
17Liverpool Clinical Laboratories, Royal Liverpool and Broadgreen University Hospitals NHS Trust, Liverpool, UK
18Department of Molecular and Clinical Pharmacology, University of Liverpool, Liverpool, UK

Global spread and evolutionary links of an epidemic Clostridium difficile strain (PCR-ribotype 027) have been noted in recent decades. However, in Japan, no outbreaks caused by type 027 have been reported to date. A total of 120 C. difficile isolates from patients at 15 hospitals during non-outbreak seasons between 2011 and 2013 as well as 18 and 21 isolates collected from two hospitals in outbreak settings in Japan, were examined. Among these 120 isolates, Japan-ribotypes smz and ysmz (subtype variant of smz) were the most predominant (39.2 %) followed by Japan-ribotype trf (15.8 %). Types smz/ysmz and trf were also concurrently predominant at two hospitals in the outbreak settings. Out of the five binary-toxin-positive isolates observed, only one was PCR-ribotype 027 and another PCR-ribotype 078. Type smz was later found to correspond to PCR-ribotype 018. High rates of
INTRODUCTION

Clostridium difficile is well known as the leading cause of healthcare-associated infectious diarrhea. The global spread of a hypervirulent strain, PCR-ribotype 027 (BI/NAP1/027), that is resistant to fluoroquinolones has been reported in recent decades, and its influence on both the incidence of C. difficile infection (CDI) and the severity of CDI has widely been acknowledged (Miller et al., 2010; Kato et al., 2010; Wilcox et al., 2012; He et al., 2013; Davies et al., 2014). Similarly, PCR-ribotype 078 was reported to be another hypervirulent strain and linked with a severe disease (Goorhuis et al., 2008; Walker et al., 2013). A surveillance study in 34 European countries indicated that infections by PCR-ribotypes 018 and 056 were significantly associated with complicated disease outcome (Bauer et al., 2011). PCR-ribotype 018 and ribotype 356, of which the fingerprinting profile is closely related to that of 018, were reported to be predominant and associated with multiple antimicrobial resistance in Italy (Freeman et al., 2015; Spigaglia et al., 2010). Also, PCR-ribotype 018 was reported to be a predominant strain in Korea (Han et al., 2014).

On the other hand, the emergence of toxin-A-negative, toxin-B-positive (A\(^-\)B\(^+\)) C. difficile has been noted at some Japanese hospitals (Komatsu et al., 2003; Sato et al., 2004; Kato et al., 2010). In the present study, we analysed C. difficile isolates collected from 15 different hospitals between 2011 and 2013 in non-outbreak settings, and from outbreaks that occurred independently in 2009 and 2010 at two hospitals in Japan.

METHODS

Bacterial strains. A total of 120 C. difficile isolates recovered from sporadic CDI cases at 15 medical facilities in 13 prefectures in Japan (hospitals A to O) in non-outbreak settings during a two-year period from April 2011 to March 2013, were examined. Between five and 10 CDI patients were chosen randomly at each hospital, and informed consent was obtained from each of them. Among the 15 hospitals investigated, hospitals L and N were previously reported as outbreak settings in 2010 and 2009, respectively, based on the incidence figures of new cases, which was more than double the averages in the previous period at these sites. In total, 21 and 18 isolates from hospital L and hospital N in outbreak periods, respectively, were available for this study. This study was approved by the Ethics Board of National Institute of Infectious Diseases (NIID), Tokyo, Japan.

PCR detecting the toxin genes and typing analysis. The presence of the non-repeating sequences of the toxin B gene (tcdB) and the repeating sequences of the toxin A gene (tcdA) was examined by PCR as described previously (Kato et al., 2005). Detection of the gene encoding the binding component of binary toxin (CDT) was performed as described by Stubbs et al. (2000). Typing analysis by PCR ribotyping was performed as described by Stubbs et al. (1999) with minor modifications (Kato et al., 2010). A new PCR ribotype was identified when two or more band differences were found from previously identified patterns (Kato et al., 2010). Isolates identified as types smz, ysmz and trf by the Japan ribotyping scheme in NIID were subject to PCR ribotyping at the Liverpool Clinical Laboratories (LCL, Royal Liverpool and Broadgreen University Hospitals, Liverpool, UK). slpA sequence typing was carried out as described previously (Kato et al., 2010). When compared with the reference libraries, isolates were assigned to distinct major groups if they had 20 or more differing amino acids, but were considered as subtypes when such differences were restricted to less than 20 (Kato et al., 2010).
Antimicrobial susceptibility testing. Isolates were tested for susceptibility to gatifloxacin, moxifloxacin, erythromycin, clindamycin, rifampicin, vancomycin and metronidazole by using Etest strips (SYSMEX-bioMérieux) according to the manufacturer’s instructions. The breakpoints used were 8 μg ml⁻¹ for gatifloxacin, moxifloxacin, erythromycin and clindamycin, 16 μg ml⁻¹ for vancomycin, and 32 μg ml⁻¹ for rifampicin and metronidazole. Breakpoints for the interpretation of susceptibility test results of moxifloxacin, clindamycin and metronidazole were available from the Clinical and Laboratory Standards Institute (CLSI, 2014). The CLSI breakpoint for anaerobes against moxifloxacin was used for the breakpoint against gatifloxacin (CLSI, 2014). The MIC results for erythromycin, rifampicin and vancomycin were interpreted as previously described (Spigaglia et al., 2011; Tenover et al., 2012).

RESULTS

Typing results of isolates recovered from non-outbreak settings

Of the 120 isolates which came from 15 hospitals in non-outbreak settings, 96 (80.0 %) isolates were A" B⁺ CDT⁻ and 19 (15.8 %) were A" B⁺ CDT⁺. The remaining 5 (4.2 %) isolates were identified as toxin-A-positive, toxin-B-positive, CDT-positive (A" B⁺ CDT⁺). These 120 isolates were assigned to 24 PCR ribotypes, and to 19 slpA sequence major types and 29 slpA sequence subtypes (Table S1 available in the online Supplementary Material). PCR-ribotype smz (A" B⁺ CDT⁻) was most disseminated and accounted for 34.2 % of isolates (41/120) (Table 1); at least one isolate of this type was found in each of the 15 hospitals examined. We found a variant of PCR-ribotype smz, whose ribotype pattern was nearly identical with that of type smz but different in more than one band (Fig. 1). Thus, we termed it type ysmz. Isolates of both smz and ysmz ribotypes were classified into the same major slpA sequence type. The PCR-ribotype ysmz was recovered only at hospital N in a non-outbreak setting in 2013, and during the outbreak period in 2009 (Table 1). Of the 120 isolates, 47 (39.2 %) were PCR-ribotype smz or ysmz, and these isolates were assigned to three slpA sequence subtypes (smz-01, smz-02 and smz-03), with subtype smz-01 being most common. All 19 A" B⁺ CDT⁺ isolates were PCR-ribotype trf (Fig. 1) and slpA sequence major type fr, and were further assigned to six different slpA sequence subtypes. Sixteen (13.3 %) and 13 (10.8 %) isolates were PCR-ribotypes 002 and 014, respectively. PCR-ribotypes smz, ysmz, trf, 002 and 014 together accounted for 79.2 % of isolates (95/120), and the remaining 25 isolates were typed into 19 and 16 different PCR ribotypes and slpA sequence types, respectively (Tables S1 and 1). Among five A" B⁺ CDT⁺ isolates, five different PCR ribotypes including types 019, 027 and 078 were identified. The PCR ribotype pattern of one isolate was nearly identical with that of the reference strain (strain CA8), characterized previously as PCR-ribotype 056 (Killage et al., 2008), but was different in more than one band (PCR-ribotype c056 in Table S1). This isolate had the slpA gene, the sequence of which was identical to that of strain CA8 (slpA sequence type y02-01).

Typing results of isolates recovered in outbreak settings

The 21 isolates from hospital L in an outbreak period were assigned to four PCR ribotypes (014, smz, trf and sc1026) and six slpA sequence types (Tables S1 and 1). Of those isolates recovered from CDI patients hospitalized in six wards during a four-week period, 13/21 (61.9 %) came from CDI patients at one ward. Out of the 13 isolates from the ward, eight, four and one were classified into PCR-ribotype smz/slpA sequence type smz-01, trf/fr-12 and trf/fr-01, respectively. The slpA sequence type fr-12 was not found among the 120 isolates obtained at the 15 hospitals, including hospital L, in non-outbreak settings. CDI due to PCR-ribotype

<table>
<thead>
<tr>
<th>Japan-ribotype</th>
<th>PCR-ribotype</th>
<th>Toxin production*</th>
<th>No. of isolates (%) recovered from 15 hospitals in non-outbreak settings</th>
<th>No. of isolates (%) in an outbreak setting at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hospital L</td>
<td>Hospital N</td>
</tr>
<tr>
<td>yoku†</td>
<td>002</td>
<td>A&quot; B⁺ CDT⁻</td>
<td>16 (13.3)</td>
<td>0</td>
</tr>
<tr>
<td>hr†</td>
<td>014</td>
<td>A&quot; B⁺ CDT⁻</td>
<td>13 (10.8)</td>
<td>5 (23.8)</td>
</tr>
<tr>
<td>smz</td>
<td>018</td>
<td>A&quot; B⁺ CDT⁻</td>
<td>41 (34.2)</td>
<td>9 (42.9)</td>
</tr>
<tr>
<td>ysmz</td>
<td>018'</td>
<td>A&quot; B⁺ CDT⁻</td>
<td>6 (5.0)</td>
<td>0</td>
</tr>
<tr>
<td>fr†</td>
<td>017</td>
<td>A&quot; B⁺ CDT⁻</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>trf</td>
<td>369</td>
<td>A&quot; B⁺ CDT⁻</td>
<td>19 (15.8)</td>
<td>6 (28.6)</td>
</tr>
<tr>
<td>Other types</td>
<td></td>
<td></td>
<td>25 (20.8)</td>
<td>1 (4.8)</td>
</tr>
<tr>
<td>Total no. isolates tested</td>
<td></td>
<td></td>
<td>120</td>
<td>21</td>
</tr>
</tbody>
</table>

† The nomenclature of Japan-ribotypes was used in previous studies (Iwashima et al., 2010; Kato et al., 2001a, b; Sawabe et al., 2007).
014 was found in the wards other than the one where smz/smz-01 and trf/fr-14 were epidemic.

Among the 18 *C. difficile* isolates recovered from six wards of hospital N during an outbreak of CDI, there were two predominant types, PCR-ribotype trf/slA sequence type fr-01 (9 isolates) and type ysmz/smz-01 (8 isolates) (Tables S1 and 1). The remaining one isolate was found to be type smz/smz-01. Both trf/fr-01 and ysmz/smz-01 isolates were disseminated across four and five distinct wards, respectively. Isolation of PCR-ribotype ysmz was restricted to hospital N and this isolate was observed in six of 10 isolates recovered during an endemic period later on.

**Analysis of isolates identified as types smz, ysmz and trf by the Japan ribotyping scheme**

Since PCR-ribotypes smz/ysmz and PCR-ribotype trf accounted for a significant proportion of clinical isolates in this and previous studies in Japan (Kato et al., 2001a, 2010; Sawabe et al., 2007; Iwashima et al., 2010), they were further analysed at LCL. We later identified Japan-ribotype smz as being PCR-ribotype 018 and replicated a minor band variation between ribotypes smz and ysmz, which was termed PCR-ribotype 018'. Since Japan-ribotype trf did not match any known reference strains from the LCL library, further investigation of the trf strain was conducted by Dr V. Hall (Public Health Wales, Cardiff, UK); a novel ribotype was assigned, here designated PCR-ribotype 369.

**Antimicrobial susceptibility results**

The MIC results of gatifloxacin, moxifloxacin, erythromycin, clindamycin, rifampicin, vancomycin and metronidazole for *C. difficile* stratified according to PCR ribotype in non-outbreak settings and in outbreak settings are shown in Tables 2 and 3, respectively. PCR-ribotypes smz and ysmz are presented together in Tables 2 and 3. Resistance to gatifloxacin, moxifloxacin, erythromycin and clindamycin was observed in 100 %, 95.7 %, 100 % and 97.9 % of PCR-ribotype smz/ysmz isolates collected from 15 hospitals, respectively. All PCR-ribotype smz/ysmz isolates from two hospitals in outbreak settings were resistant to gatifloxacin, moxifloxacin, erythromycin and clindamycin. Among 19 trf isolates from 15 hospitals in non-outbreak settings, 17 (89.5 %) and 13 (68.4 %) were resistant to gatifloxacin and moxifloxacin, respectively. These 19 trf isolates were all resistant to erythromycin and clindamycin. All of the 15 trf isolates (6 from hospital L and 9 from hospital N) recovered from outbreak settings were resistant to gatifloxacin. Among six trf isolates from hospital L in an outbreak period, MICs of moxifloxacin were 6 µg ml⁻¹ and 8 µg ml⁻¹ in four and two isolates, respectively. Of nine trf isolates from hospital N in an outbreak period, eight were resistant to moxifloxacin; the MIC of the remaining one was 6 µg ml⁻¹. High resistance to both erythromycin and clindamycin was present in the 15 trf isolates prevalent at hospitals L and N; the MIC value of all 15 isolates was more than 256 µg ml⁻¹. Resistance to gatifloxacin, moxifloxacin, erythromycin and clindamycin was observed sporadically in 002 isolates examined in this study. In PCR-ribotype 014 isolates recovered in both non-outbreak settings and outbreak settings, resistance to gatifloxacin, moxifloxacin, erythromycin and clindamycin was less common. Rifampicin resistance (MIC >32 µg ml⁻¹) was observed in only one isolate, which was typed as PCR-ribotype og39/slA sequence type og39-01. This isolate was highly resistant to erythromycin and clindamycin, but susceptible to gatifloxacin and moxifloxacin. All 159 isolates examined had
Table 2. MIC results of *C. difficile* isolates recovered in non-outbreak settings at 15 hospitals

<table>
<thead>
<tr>
<th>PCR ribotype</th>
<th>No. of isolates</th>
<th>Measure</th>
<th>MIC results (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gatofloxacin</td>
<td>Moxifloxacin</td>
</tr>
<tr>
<td>002</td>
<td>16</td>
<td>Range</td>
<td>0.75–&gt;32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>&gt;32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>&gt;32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% R&lt;sup&gt;*&lt;/sup&gt;</td>
<td>75.0</td>
</tr>
<tr>
<td>014</td>
<td>13</td>
<td>Range</td>
<td>0.75–&gt;32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% R&lt;sup&gt;*&lt;/sup&gt;</td>
<td>23.1</td>
</tr>
<tr>
<td>018 (smz)/018&lt;sup&gt;+&lt;/sup&gt; (ysmz)&lt;sup&gt;†&lt;/sup&gt;</td>
<td>47</td>
<td>Range</td>
<td>8–&gt;32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>&gt;32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>&gt;32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% R&lt;sup&gt;*&lt;/sup&gt;</td>
<td>100</td>
</tr>
<tr>
<td>369 (trf)</td>
<td>19</td>
<td>Range</td>
<td>6–&gt;32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>&gt;32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% R&lt;sup&gt;*&lt;/sup&gt;</td>
<td>89.5</td>
</tr>
<tr>
<td>Other types</td>
<td>25</td>
<td>Range</td>
<td>0.38–&gt;32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% R&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.0</td>
</tr>
</tbody>
</table>

*% R, percentage of resistant isolates.

†PCR ribotypes 018 (smz) and 018<sup>+</sup> (ysmz) are presented together.
Table 3. MIC results of *C. difficile* isolates recovered from two hospitals in outbreak settings

<table>
<thead>
<tr>
<th>PCR ribotype</th>
<th>No. of isolates</th>
<th>Measure</th>
<th>MIC results (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gatofloxacin</td>
<td>Moxifloxacin</td>
</tr>
<tr>
<td><em>C. difficile</em> isolates from hospital L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>014</td>
<td>5</td>
<td>Range</td>
<td>1–1.5</td>
</tr>
<tr>
<td>MIC₉₀</td>
<td>1</td>
<td>0.75</td>
<td>1</td>
</tr>
<tr>
<td>MIC₉₀</td>
<td>1.5</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>% R*</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>018 (smz)</td>
<td>9</td>
<td>Range</td>
<td>&gt;32</td>
</tr>
<tr>
<td>MIC₉₀</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;256</td>
</tr>
<tr>
<td>MIC₉₀</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;256</td>
</tr>
<tr>
<td>% R</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>369 (trf)</td>
<td>6</td>
<td>Range</td>
<td>12–16</td>
</tr>
<tr>
<td>MIC₉₀</td>
<td>12</td>
<td>6</td>
<td>&gt;256</td>
</tr>
<tr>
<td>MIC₉₀</td>
<td>16</td>
<td>8</td>
<td>&gt;256</td>
</tr>
<tr>
<td>% R</td>
<td>100</td>
<td>33.3</td>
<td>100</td>
</tr>
<tr>
<td>sc1026</td>
<td>1</td>
<td>MIC</td>
<td>1</td>
</tr>
<tr>
<td><em>C. difficile</em> isolates from hospital N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>018 (smz)/018’ (ysmz)†</td>
<td>9</td>
<td>Range</td>
<td>&gt;32</td>
</tr>
<tr>
<td>MIC₉₀</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;256</td>
</tr>
<tr>
<td>MIC₉₀</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;256</td>
</tr>
<tr>
<td>% R</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>369 (trf)</td>
<td>9</td>
<td>Range</td>
<td>8–&gt;32</td>
</tr>
<tr>
<td>MIC₉₀</td>
<td>24</td>
<td>8</td>
<td>&gt;256</td>
</tr>
<tr>
<td>MIC₉₀</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;256</td>
</tr>
<tr>
<td>% R</td>
<td>100</td>
<td>88.9</td>
<td>100</td>
</tr>
</tbody>
</table>

*% R, percentage of resistant isolates.
†PCR ribotypes 018 (smz) and 018’ (ysmz) are presented together.
vancomycin MIC values distributed over a narrow range (0.19–1 μg ml⁻¹). The metronidazole MIC was ≤0.5 μg ml⁻¹ for all the isolates. One isolate was identified as PCR-ribotype 027 and was susceptible to all seven antimicrobial agents tested. The PCR-ribotype 078 isolate recovered in this study was highly resistant to erythromycin, but susceptible to the other six agents.

**DISCUSSION**

PCR-ribotype smz designated by the Japan typing scheme was found to correspond to PCR-ribotype 018 in the present study. PCR-ribotypes 018 (smz)/018' (ysmz) were identified as the most common circulating strains (39.2 %), and were disseminated across 15 hospitals examined. Also, types 018 (smz) and 018' (ysmz) caused outbreaks at two hospitals, highlighting a major epidemiological role of them in both epidemic and endemic CDI in Japan. Of 365 isolates obtained from 26 European countries in 2008, 23 (6 %) were assigned as PCR-ribotype 018, 19 of which were recorded in Italy (Bauer et al., 2011). In a more recent study from 20 European countries, PCR-ribotype 018 was one of the ten most common types, although distribution of 018 was not mentioned (Davies et al., 2014). In Italy, PCR-ribotype 018 C. difficile was predominant in 2007 and 2008, superseding an other type (PCR-ribotype 126), which was the most predominant strain until 2005 (Spigaglia et al., 2010); from 2012 to 2014, 20 % of isolates from Italy were identified as type 018 (Freeman et al., 2015). A recent prospective study showed that PCR-ribotype 018 was also the predominant strain (48.1%) at three hospitals in Korea from 2011 to 2012 (Han et al., 2014). Conversely, it was documented that type 018 was not prevalent in England (Wilcox et al., 2012) and the USA (Tickler et al., 2014). In Japan, there have been historical reports suggesting a high prevalence of PCR-ribotype 018 since the 1990s, when it was already identified as the most prevalent strain at three Japanese hospitals between 1996 and 1999 (Kato et al., 2001a) and at four other sites between 2003 and 2007 (Kato et al., 2010). An independent epidemiological study at another hospital in Japan showed that PCR-ribotype 018 replaced PCR-ribotype 014 as the most predominant strain over a five-year period from 2000 to 2004 (Sawabe et al., 2007). A similar shift was observed at a hospital in Korea, where PCR-ribotype 018 has been identified since 2006, and became more prevalent than PCR-ribotype 001 over a 10-year period between 2000 and 2009 (Lee et al., 2014). Interestingly, in a healthy volunteer study in Japan, 1234 individuals were examined and 94 (7.6 %) were found to be colonized by C. difficile, but none of them carried type 018 isolates in their intestinal tract (Kato et al., 2001b). Isolation of PCR-ribotype 018 C. difficile from edible bivalve molluscs (Pasquale et al., 2012) and poultry (Janezic et al., 2014) has been reported, suggesting that food intake may constitute an important route of C. difficile infection. Further molecular studies on community-acquired CDI and food surveillance are required in Japan.

In the present study, we identified PCR-ribotype 018' (ysmz), which had a ribotype pattern shared by most bands of type 018 (smz), and all type 018' isolates tested in the present study displayed slpA sequence type smz-01. The type 018' strain was exclusive to hospital N and was recovered in both an outbreak period (2009) and a non-outbreak setting (2012–2013), suggesting that the 018' might have persisted within CDI inpatients, asymptomatic carriers and/or in inanimate environments at hospital L for years. More recently, the emergence of PCR-ribotype 356 has been noted in Italy, and it was postulated that this is likely a strain subtype that may have evolved from the main PCR-ribotype 018 lineage (Freeman et al., 2015). Albeit ribotype profiles of types 018' (ysmz) and 356 have not been compared directly, it is epidemiologically significant that strain variants similar to ribotype 018 emerged in independent areas where this strain was predominant.

An early report showed that PCR ribotype 018 has been an endemic strain since the 1990s in Japan (Kato et al., 2001a), suggesting that 018 and its variants might have spread from Asia, including Japan, to other countries. More comprehensive studies at a whole genome sequencing level on types 018/018' in Asia and type 018/356 in Europe are required to unveil its evolution pattern and potential of acquired functional mechanisms in order to understand their global spread and epidemiology.

High rates of resistance to gatifloxacin, moxifloxacin, erythromycin and clindamycin in both PCR-ribotype 018 and 018' isolates were observed, although they both remained susceptible to rifampicin. In Italy, resistance against fluoroquinolones in type 018 isolates has been documented (Spigaglia et al., 2010; Freeman et al., 2015).
In a Korean study, eight isolates belonging to type 018 were tested and all of them had high overall resistance against several antibiotics including moxifloxacin, erythromycin and clindamycin (Lee et al., 2014). Sawabe et al. (2007) reported that all type 018 isolates at their hospital in Japan were resistant to gatifloxacin and moxifloxacin, as well as clindamycin, and that the noticeable shift in endemic strain from type 014 to type 018 may have been related to the introduction of gatifloxacin to their hospital in 2002. He et al. (2013) documented that the acquisition of resistance to commonly used antibiotics (fluoroquinolones) is a major feature of the continued evolution and persistence of C. difficile BI/NAP1/027 in healthcare settings. In this study, we found only one type 027 isolate, which was susceptible to new fluoroquinolones. In Japan, while some reports have shown sporadic cases including one with fulminant colitis due to PCR-ribotype 027 C. difficile (Kato et al., 2007; Sawabe et al., 2007; Nishimura et al., 2014), no outbreaks associated with type 027 have been reported so far. It is unknown why the nosocomial spread of 027 C. difficile resistant to fluoroquinolones has not been found in Japan, where type 018 resistant to fluoroquinolones has been distributed to a number of hospitals. Similarly, PCR-ribotype 078 C. difficile was isolated from only one patient in the present study, while this strain has been reported to be recovered more frequently from animals in Japan (Niwa et al., 2013; Usui et al., 2014). The lower frequency of isolation, either of PCR-ribotype 027 or 078 from CDI in human in Asian countries including Japan (Cheng et al., 2011; Han et al., 2014), may reflect differences in the intestinal ecosystem and/or immunological responses between Asian populations and those of countries where 027 or 078 have become predominant. Freeman et al. (2015) documented that all ribotype 018 and 356 isolates obtained from Italy were resistant to rifampicin, but rifampicin resistance was not observed in type 018/018’ isolates examined in our study. Also, Freeman et al. (2015) showed that vancomycin MICs were higher among 018 and 356 with geometric mean MICs of 2.00 μg ml⁻¹ and 2.28 μg ml⁻¹, respectively, compared with those of the remaining common ribotypes; geometric mean MICs of metronidazole were elevated in ribotype 027 (1.42 μg ml⁻¹) and ribotype 356 (0.61 μg ml⁻¹) isolates (Freeman et al., 2015). Tickler et al. (2014) observed reduced susceptibility to vancomycin in 39.2 % of 027 isolates collected from US hospitals. In our study, none of the isolates tested including type 018/018’ showed reduced susceptibility to vancomycin and metronidazole. The potential emergence of increasing resistance to vancomycin and metronidazole may warrant further monitoring of MICs of the agents especially in type 018/018’ isolates in Japan.

In contrast, the emergence of A⁻B⁺ isolates, which have 1.8 kbp deletions in the repeating sequences in tcdA and belong to toxinotype VIII, has been reported in Japan as well as the Netherlands, Ireland, Poland, Korea, China and Argentina (Kuijper et al., 2001; Komatsu et al., 2003; Drudy et al., 2007; Shin et al., 2008; Goorhuis et al., 2009, Kato et al., 2010; Pituch et al., 2011; Hawkey et al., 2013). The reports from all these countries except Japan documented that the vast majority of these A⁻B⁺ isolates were found to be of PCR-ribotype 017 (Kuijper et al., 2001; Drudy et al., 2007; Goorhuis et al., 2009; Pituch et al., 2011; Hawkey et al., 2013; Lee et al., 2014). PCR-ribotype 017 was identified in 4 % of isolates from 34 European countries (Davies et al., 2014) and 2.2 % of isolates from 32 hospitals in the USA (Tickler et al., 2014). Although we have reported the isolation of PCR-ribotype 017 from sporadic cases (Kato et al., 2001b, 2010; Iwashima et al., 2010) in Japan, notably, all 34 A⁻B⁺ isolates collected in the present study were typed as Japan-ribotype trf, which corresponded neither to type 017 nor 047, and was newly assigned as PCR-ribotype 369. In this study, type 369 was the second most frequent ribotype (15.8 %) among 120 isolates from 15 hospitals in non-outbreak settings, and was also prevalent at two hospitals during outbreak periods. The type 369 strain was reported to be blamed for outbreaks that occurred at Japanese hospitals in 2000 and 2001 (Komatsu et al., 2003; Sato et al., 2004); ribotype 369 has been identified as an epidemic strain since the early 2000s in Japan. Both PCR-ribotype 017 and 369 isolates tested had identical deletions at the repeating sequences in tcdA and a nonsense mutation introducing a stop codon at amino acid position 47 of tcdA (data not shown), and were classed as slda sequence major type fr. This suggests that types 017 and 369 are not phylogenetically distant to each other and they may share a common recent ancestor, though deep genome sequencing has not been performed yet. Evolutionary comparative analysis and determination of exclusive factors to each strain will allow understanding of the preferential establishment of one or another, such as of 369 in Japan and type 017 in Europe. High resistance against erythromycin and clindamycin was observed in all type 369 isolates tested, which is consistent with the previous reports on type 017 strains (Kuijper et al., 2001; Pituch et al., 2011; Lee et al., 2014; Freeman et al., 2015). In addition, 94.1 % (32/34) and 67.6 % (23/34) of type 369 isolates examined by this study were resistant to gatifloxacin and moxifloxacin, respectively. Resistance against new fluoroquinolones in PCR-ribotype 017 was also reported from other countries (Pituch et al., 2011; Spigaglia et al., 2011; Lee et al., 2014; Freeman et al., 2015), indicating the need of escalating attention to isolates belonging to toxinotype VIII, which have been showing multi-resistance tendencies.

It was reported that PCR-ribotype 002 constituted approximately 5 % of isolates from European countries in 2008, and 3.5 % of isolates from US hospitals between 2011 and 2013 (Bauer et al., 2011; Tickler et al., 2014). The significant increase in the prevalence of type 002 from 3 % (2007–2008) to 6 % (2009–2010) was documented in England (Wilcox et al., 2012). Moreover, PCR-ribotype 002 was reported to be the most prevalent type (10.1 %) in a healthcare region in Hong Kong (Cheng et al., 2011). In Japan, PCR-ribotype 002 was the third most frequently
found (13.3 %) in the present study, and a three-year investigation at a university hospital in Japan showed that type 002 was one of the three most prevalent types (19.7 %) (Iwashima et al., 2010). In addition, Tenover et al. (2010) reported that the sensitivity of the enzyme immunoassay (EIA) was significantly lower for detecting toxins from specimens of CDI infected by ribotype 002, suggesting that these cases may be overlooked when diagnosed by EIA for toxins only. More studies of worldwide variation and differences in prevalence and disease severity by type 002 are required.

PCR-ribotype 014 has been reported to be one of the most common PCR ribotypes in the world (Kato et al., 2001a, 2010; Iwashima et al., 2010; Bauer et al., 2011; Cheng et al., 2011; Wilcox et al., 2012; Davies et al., 2014; Freeman et al., 2014; Han et al., 2014; Tickler et al., 2014). In the present study, 10.8 % (13/120) of the isolates recovered in non-outbreak settings and 19.0 % (4/21) of the isolates recovered in an outbreak period at hospital L were typed as PCR-ribotype 014. Moreover, a previous study showed that PCR-ribotype 014 was in fact the most frequently isolated strain from healthy Japanese individuals; of 94 individuals positive for C. difficile-culture, 17 of them (18.0 %) had this type (Kato et al., 2001b). Janezic et al. (2014) documented that PCR-ribotype 014 was the second most prevalent strain following type 078 among animal isolates and also had a broader range of animal hosts. In our study, the resistance of PCR-ribotype 014 to gatifloxacin, moxifloxacin, erythromycin and clindamycin was less common, consistent with the results of other reports (Tickler et al., 2014; Freeman et al., 2015). The ability to colonize a variety of hosts including humans may be responsible for its relatively high nosocomial and community prevalence (Janezic et al., 2014).

Our study showed no difference in the mean age of CDI patients due to strains 018, 369, 002 or 014. Also, we found no significant correlation between the CDI treatments and PCR ribotypes. Since only limited data about clinical features of patients, such as presence of comorbidities, symptoms and outcomes including recurrences was available, neither potential risk factors for CDI due to predominant types nor specific correlation between outcomes and type differences could be clarified in this study. It was reported that PCR-ribotype 018 was significantly associated with complicated disease outcome (Bauer et al., 2011). Prospective studies are needed to elucidate the involvement of these types with CDI progression and clinical outcomes.

In summary, PCR-ribotypes 018 (smz)/018’ (ysmz), 369 (trf), 002 and 014 were identified as the major types circulating in Japan. Particularly, types 018/018’ and 369 were found to be prevalent and associated with both epidemic and endemic CDI in Japan. A European study demonstrated that increased awareness of CDI and the use of optimal testing methods could reduce the dissemination of epidemic strains (Davies et al., 2014). The high epidemiicity of ribotypes 018 and 369 and a lower diversity of ribotypes observed in the present study may reflect the insufficient awareness of CDI and a suboptimal test-density in Japan. Further studies are warranted to understand the epidemiological relevance and the role of these strains in relation to their spread and prevalence nationwide and globally.

ACKNOWLEDGEMENTS

We thank Dr V. Hall (Public Health Wales, Cardiff, UK) for analysing ribotype 369 (trf) isolate and Dr S. Suzuki (National Institute of Infectious Diseases, Tokyo, Japan) for her helpful comments. The technical assistance of S. Yonamine, K. Kai, Y. Taki, and Y. Yoshimura is gratefully acknowledged. This work was supported by a grant (Research Program on Emerging and Re-emerging Infectious Diseases) from Japan Agency for Medical Research and Development, AMED. The authors declare no conflicts of interest.

REFERENCES


Han, S. H., Kim, H., Lee, K., Jeong, S. J., Park, K. H., Song, J. Y., Seo, Y. B., Choi, J. Y., Woo, J. H. & other authors (2014). Epidemiology and


Dear Authors,

Please find enclosed a proof of your article for checking.

When reading through your proof, please check carefully authors’ names, scientific data, data in tables, any mathematics and the accuracy of references. Please do not make any unnecessary changes at this stage. All necessary corrections should be marked on the proof at the place where the correction is to be made; please mark up the correction in the PDF and return it to us (see instructions on marking proofs in Adobe Reader).

Any queries that have arisen during preparation of your paper for publication are listed below and indicated on the proof.

Please provide your answers when returning your proof.

Please return your proof by email (sgmprod@charlesworth-group.com) within 2 days of receipt of this message.

<table>
<thead>
<tr>
<th>Query no</th>
<th>Query</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Please check the use of a prime in PCR-ribotype 018’ and change to an apostrophe in the text, figure legend and tables if necessary.</td>
</tr>
<tr>
<td>2</td>
<td>Please check that the date in reference citation Tenover et al. (2010) is correct.</td>
</tr>
<tr>
<td>3</td>
<td>Please check the number of isolates recovered in an outbreak period at hospital L and typed as PCR-ribotype 014.</td>
</tr>
</tbody>
</table>
Ordering reprints for SGM journals

As a result of declining reprint orders and feedback from many authors who tell us they have no use for reprints, SGM no longer provides free reprints to corresponding authors; instead, corresponding authors will receive two emails:

i) An email including a link to download the published PDF of their paper. You can forward this link to co-authors or others, and they can also use it to download the published PDF. The link can be used up to 25 times. This email will be sent out at around the time your article is published online.

ii) An email including a link to the SGM Reprint Service. You can forward this email to your co-authors if you wish, so that they can order their own reprints directly, or to your finance or purchasing department, if orders are placed centrally. This email will be sent out at around the time that your article is finalized for printing.

When you click on the link in this second email, you will be taken to an order page to place your reprint order. Like most online ordering sites, it is necessary to set up an account and provide a delivery address while placing your order, if you do not already have an account. Once an account and delivery address have been set up, these details will be stored by the system for use with future orders. Payments can be made by credit card, PayPal or purchase order.

As reprint orders are despatched by courier, there is a charge for postage and packing.

SUMMARY

- You can create or update your reprint account at any time at http://sgm-reprints.charlesworth.com/
- You will be sent an email when the reprints of this paper are ready for ordering
- You cannot order reprints of this paper before this email has been sent, as your paper will not be in the system
- Reprints can be ordered at any time after publication
- You will also receive an email with a link to download the PDF of your published paper

The reprint ordering details will be emailed to the author listed as the corresponding author on the journal’s manuscript submission system. If your paper has been published (the final version, not the publish-ahead-of-print version) but you have not received this notification, email reprints@sgm.ac.uk quoting the journal, paper number and publication details.

If you have any questions or comments about the reprint-ordering system or about the link to your published paper, email reprints@sgm.ac.uk