

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

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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 2010 and 2009, respectively, in outbreak periods 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

resistance against gatifloxacin, moxifloxacin, erythromycin and clindamycin were observed in the PCR-ribotype 018 isolates. Interestingly, all trf isolates were toxin-A-negative, toxin-B-positive, but they did not correspond to PCR-ribotype 017, thus being assigned a new ribotype (PCR-ribotype 369). In conclusion, PCR-ribotypes 018 (smz) and 369 (trf) were identified as major circulating strains in both outbreak and non-outbreak settings in Japan. Given their epidemiological relevance, molecular investigations are warranted to clarify potential evolutionary links with related strains found elsewhere, such as PCR-ribotypes 018 and 017 from Europe and North America.

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INTRODUCTION

Clostridium difficile is well known as the leading cause of healthcare-associated infectious diarrhoea. 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; 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 in the Netherlands, Ireland, Poland, Korea, China and Argentina (Kuijper *et al.*, 2001; Drudy *et al.*, 2007; Pituch *et al.*, 2011; Shin *et al.*, 2008; Goorhuis *et al.*, 2009; Hawkey *et al.*, 2013), and in some of these reports the resistance of PCR-ribotype 017 (A⁻B⁺) to clindamycin was documented (Kuijper *et al.*, 2001; Goorhuis *et al.*, 2009; Pituch *et al.*, 2011). It was reported that differences in strain types have an impact not only on epidemiology but also on course of treatment and laboratory diagnosis (Louie *et al.*, 2011; Tenover *et al.*, 2010). Hence, a number of reports have

emphasized the importance of local surveillance of both endemic and epidemic strains.

In Japan, we have reported that a specific *C. difficile* type strain, PCR-ribotype smz, has been dominant at numerous hospitals since the 1990s (Kato *et al.*, 2001a, 2010; Sawabe *et al.*, 2007; Iwashima *et al.*, 2010). In addition, frequent isolation of 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).

Abbreviations: CDI, *Clostridium difficile* infection; CDT, binary toxin; LCL, Liverpool Clinical Laboratories

The GenBank/EMBL/DBJ accession numbers (*slpA* sequence type) for the *slpA* genes reported in this study are AB249985 (gr-02), LC003024 (yok-05), AB534595 (hr-04), AB704921 (fr-07), AB704922 (fr-08), AB704920 (fr-10), AB761399 (fr-11), AB819625 (fr-12), LC003023 (fr-16), LC066519 (ar-01), AB533281 (08162-01), LC066520 (j52-01), AB770148 (ac12056-01), AB770151 (cc12078-01), AB819404 (y05-02) and AB770145 (y02-01).

One supplementary table is available with the online Supplementary Material.

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 (Killgore *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 1. Distribution of prevalent PCR ribotypes recovered from 15 hospitals in non-outbreak settings and 2 hospitals in outbreak settings

| Japan-ribotype | PCR-ribotype | Toxin production* | No. of isolates (%) recovered from 15 hospitals in non-outbreak settings | No. of isolates (%) in an outbreak setting at: | |
|------------------------------|--------------|--|--|--|------------|
| | | | | Hospital L | Hospital N |
| yok† | 002 | A ⁺ B ⁺ CDT ⁻ | 16 (13.3) | 0 | 0 |
| hr† | 014 | A ⁺ B ⁺ CDT ⁻ | 13 (10.8) | 5 (23.8) | 0 |
| smz | 018 | A ⁺ B ⁺ CDT ⁻ | 41 (34.2) | 9 (42.9) | 1 (5.6) |
| ysmz | 018' | A ⁺ B ⁺ CDT ⁻ | 6 (5.0) | 0 | 8 (44.4) |
| fr† | 017 | A ⁻ B ⁺ CDT ⁻ | 0 | 0 | 0 |
| trf | 369 | A ⁻ B ⁺ CDT ⁻ | 19 (15.8) | 6 (28.6) | 9 (50.0) |
| Other types | | | 25 (20.8) | 1 (4.8) | 0 |
| Total no. of isolates tested | | | 120 | 21 | 18 |

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

†The nomenclature of Japan-ribotypes was used in previous studies (Iwashima *et al.*, 2010; Kato *et al.*, 2001a, b; Sawabe *et al.*, 2007).

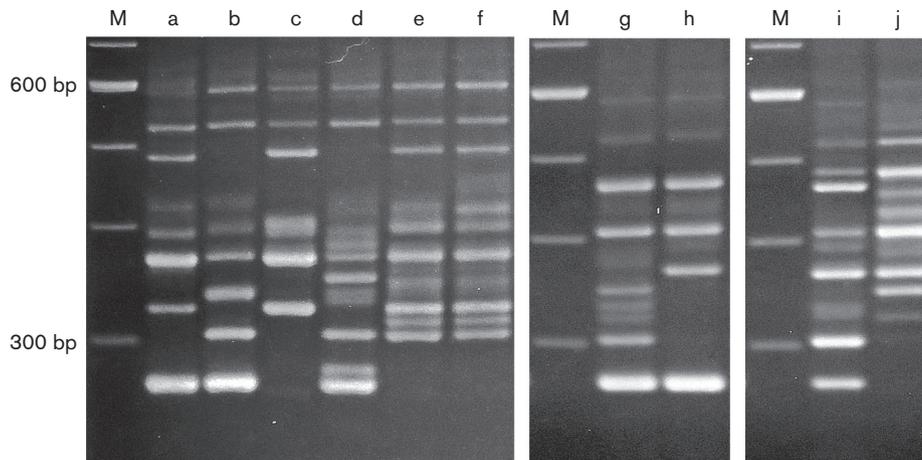


Fig. 1. Representative PCR ribotype patterns of *C. difficile* isolates. Lanes: M, 100 bp ladder as a molecular mass marker; a, PCR ribotype og39; b, type 001; c, type 002; d, type 014; e, type 018' (ysmz); f, type 018 (smz); g, type 017; h, type 369 (trf); i, type 027; j, type 078.

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/*slpA* 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 $6 \mu\text{g ml}^{-1}$ and $8 \mu\text{g ml}^{-1}$ 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 $6 \mu\text{g ml}^{-1}$. 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 $256 \mu\text{g ml}^{-1}$. 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 ($\text{MIC} > 32 \mu\text{g ml}^{-1}$) was observed in only one isolate, which was typed as PCR-ribotype og39/*slpA* 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

| PCR ribotype | No. of isolates | Measure | MIC results ($\mu\text{g ml}^{-1}$) | | | | | | | | | |
|------------------------|-----------------|-------------------|---------------------------------------|--------------|--------------|-------------|-------------------|------------|---------------|--|--|--|
| | | | Gatifloxacin | Moxifloxacin | Erythromycin | Clindamycin | Rifampicin | Vancomycin | Metronidazole | | | |
| 002 | 16 | Range | 0.75->32 | 0.75->32 | 0.38->256 | 3-128 | ≤ 0.002 | 0.19-0.5 | | | | |
| | | MIC ₅₀ | >32 | >32 | 0.75 | 6 | ≤ 0.002 | 0.38 | | | | |
| | | MIC ₉₀ | >32 | >32 | >256 | 128 | ≤ 0.002 | 0.5 | | | | |
| | | % R* | 75.0 | 56.3 | 12.5 | 12.5 | 0 | 0 | | | | |
| 014 | 13 | Range | 0.75->32 | 0.5->32 | 0.25->256 | 3->256 | ≤ 0.002 | 0.13-0.5 | | | | |
| | | MIC ₅₀ | 1 | 1 | 0.75 | 3 | ≤ 0.002 | 0.38 | | | | |
| | | MIC ₉₀ | 8 | 8 | 1 | 6 | ≤ 0.002 | 0.5 | | | | |
| | | % R | 23.1 | 23.1 | 15.4 | 7.7 | 0 | 0 | | | | |
| 018 (smz)/018' (ysmz)† | 47 | Range | 8->32 | 6->32 | >256 | 4->256 | ≤ 0.002 | 0.13-0.5 | | | | |
| | | MIC ₅₀ | >32 | 24 | >256 | >256 | ≤ 0.002 | 0.38 | | | | |
| | | MIC ₉₀ | >32 | >32 | >256 | >256 | ≤ 0.002 | 0.5 | | | | |
| | | % R | 100 | 95.7 | 100 | 97.9 | 0 | 0 | | | | |
| 369 (trf) | 19 | Range | 6->32 | 6->32 | >256 | 64->256 | ≤ 0.002 | 0.13-0.5 | | | | |
| | | MIC ₅₀ | 12 | 8 | >256 | >256 | ≤ 0.002 | 0.38 | | | | |
| | | MIC ₉₀ | >32 | >32 | >256 | >256 | ≤ 0.002 | 0.75 | | | | |
| | | % R | 89.5 | 68.4 | 100 | 100 | 0 | 0 | | | | |
| Other types | 25 | Range | 0.38->32 | 0.38-16 | 0.38->256 | 2->256 | ≤ 0.002 ->32 | 0.19-1 | | | | |
| | | MIC ₅₀ | 1 | 1 | 1 | 6 | ≤ 0.002 | 0.5 | | | | |
| | | MIC ₉₀ | 1.5 | 1.5 | >256 | >256 | ≤ 0.002 | 0.75 | | | | |
| | | % R | 4.0 | 4.0 | 32.0 | 32.0 | 4.0 | 0 | | | | |

*% 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.

CDI due to predominant PCR ribotypes

The mean age of CDI patients due to PCR-ribotypes smz/smz, trf, 014 and 002 was 77.4, 77.6, 71.9, and 74.0 years old, respectively, and the difference in the four types was not significant. The data for CDI therapy was available in 153 of the 159 patients. While 31 (20.3 %) patients had no antibiotic treatment, 74 (48.4 %), 43 (28.1 %) and 5 (3.3 %) were treated with vancomycin, metronidazole and both, respectively. No significant correlation was found between the CDI treatments and PCR-ribotypes (data not shown). Ten of the 159 patients underwent an endoscopic examination and pseudomembranous colitis (PMC) was found in three patients, who were infected by PCR-ribotypes smz, 002 or 001. Two patients suffered from CDI with severe complications; one patient with type smz died of CDI and a pathological autopsy revealed PMC, and another patient with type 002 survived after emergency colectomy and was diagnosed with PMC during the surgery.

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 *slpA* 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 epidemicity 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.

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