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Typing of *Clostridium difficile* isolates endemic in Japan by sequencing of *slpA* and its application to direct typing

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A typing system for *Clostridium difficile* using sequencing of the surface-layer protein A encoding gene (*slpA*) was evaluated and used to analyse clinical isolates in Japan. A total of 160 stool specimens from symptomatic patients in Japan was examined and 87 *C. difficile* isolates were recovered. *slpA* sequence typing was found to have reliable typability and discriminatory power in comparison with PCR ribotyping, and the typing results were highly reproducible and comparable. *slpA* sequence typing was used to type *C. difficile* in DNA extracted directly from stool specimens. Among the 90 stool specimens in which direct typing results were obtained, 77 specimens were positive for *C. difficile* culture, and typing results from isolated strains agreed with those from direct typing in all 77 specimens. The *slpA* sequence type smz was dominant at all four hospitals examined, and this endemic type was detected by culture and/or direct typing in 61 (62 %) of 99 stool specimens positive for toxic culture and/or direct *slpA* sequence typing. Comparison of epidemic strains reported throughout the world revealed one isolate identified as *slpA* sequence type gc8, which was found to correspond to PCR ribotype 027 (BI/NAP1/027), whereas no isolates were found with the *slpA* gene identical to that of PCR ribotype 078 strain. *slpA* sequence typing is valuable for comparison of *C. difficile* strains epidemic in diverse areas because the typing results are reproducible and can easily be shared. In addition, *slpA* sequence typing could be applied to direct typing without culture.

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INTRODUCTION

Clostridium difficile is one of the important organisms causing healthcare-associated infections. Specific strains have been documented to cause multiple outbreaks (Kato *et al.*, 2001; McDonald *et al.*, 2005; Samore *et al.*, 1997), and patients infected with particular strains are more likely to develop severe disease (Barbut *et al.*, 2007; Goorhuis *et al.*, 2007, 2008), suggesting that strain differences play some role in the pathogenicity of this organism. Recent reports documented that a variant strain, characterized as restriction endonuclease analysis (REA) type BI, PFGE type NAP1 and PCR ribotype 027, caused a large number of outbreaks in North America and Europe (Kuijper *et al.*, 2008; McDonald *et al.*, 2005; Warny *et al.*, 2005). In addition, PCR ribotype 078 has been noted as another hypervirulent strain, and has been recovered not only from calves and pigs (Keel *et al.*, 2007) but also from humans (Goorhuis *et al.*, 2008). Among numerous schemes for typing *C. difficile*, PCR ribotyping and PFGE typing are widely used in Europe and North America to identify epidemic strains. However, it is not easy to share typing results by these schemes, which depend on banding-pattern

Abbreviations: CDI, *Clostridium difficile* infection; CDT, *Clostridium difficile* binary toxin; REA, restriction endonuclease analysis.

The GenBank/EMBL/DDBJ accession numbers (*slpA* sequence type) for the *slpA* genes reported in this study are AB259785 (ar-02), AB258978 (cr-01), AB258979 (cr-02), AB258980 (cr-03), AB236153 (fr-01), AB249984 (gr-01), AB231583 (hr-01), AB258982 (kr-02), AB258981 (kr-03), AB239686 (xr-01), AB239685 (xr-02), AB261625 (xr-03), AB180242 (smz-01), AB181350 (smz-02), AB256018 (smz-04), AB240196 (yok-01), AB257283 (yok-02), AB236725 (hj2-01), AB236726 (j41-01), AB258983 (gc11-01), AB259787 (og39-01), AB538230 (y05-01), AB259786 (t25-01), AB269265 (g13-01), AB249986 (gc8-01) and AB470267 (078-01).

analysis among multiple laboratories. In the present study, a typing method involving the sequencing of the gene encoding the surface-layer protein A (*slpA*) was evaluated. Previous reports have documented that the low-molecular-mass peptide of the surface-layer protein varies among *C. difficile* isolates (Calabi & Fairweather, 2002; Eidhin *et al.*, 2006), and a variation of the gene has been used for typing *C. difficile* (Karjalainen *et al.*, 2002; Kato *et al.*, 2005a). In this study, clinical isolates from Japan were analysed by sequencing of *slpA* and the method was applied to type *C. difficile* from DNA extracted directly from stool specimens.

METHODS

Bacterial strains and stool specimens. The reference strains of F (ATCC 43598), G (ATCC 43599), H (ATCC 43600) (Delmee *et al.*, 1986) were obtained from the ATCC. The GAI 97660 strain was used as a reference strain for the PCR ribotype smz, *slp*A sequence type smz and serogroup JP (Kato *et al.*, 2001, 2005a). Included in the present study were strains US36 (REA type J/PCR ribotype 001), NL8 (REA type Y/PCR ribotype 014), US37 (REA type G/PCR ribotype 002), US42 (REA type BI/PFGE type NAP1/PCR ribotype 027; Killgore *et al.*, 2008) and UMCG12(3) (PCR ribotype 078; Goorhuis *et al.*, 2008). Stool specimens were obtained with the informed consent of patients who were hospitalized from 2003 to 2007 with a diagnosis of antibiotic-associated diarrhoea or colitis. A total of 147 stool specimens from patients admitted to four hospitals (A, B, C and D) and 13 specimens from sporadic cases from six other hospitals in Japan were tested. Hospitals A and D were located in different cities of the same prefecture (Gifu), whilst hospitals B and C were in different prefectures, Aichi and Nagano, respectively. The stool specimens were frozen at −80 °C until transportation, and were tested at the National Institute of Infectious Diseases, Tokyo, Japan.

Culture. *C. difficile* was isolated on cycloserine–cefoxitin–mannitol agar (Nissui Pharmaceutical) from stool specimens, which were treated with alcohol for spore selection, and identified as described previously (Kato *et al.*, 1998). 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.*, 1998, 1999). PCR detection of the gene encoding the binding component of C. *difficile* binary toxin (CDT) was performed as described by Stubbs *et al.* (2000).

DNA extraction. DNA extraction from cultured isolates for PCR ribotyping and *slpA* sequence typing was performed using a High Pure PCR template preparation kit (Roche Diagnostics) according to the manufacturer's instructions. DNA was extracted directly from stool specimens using a QIAamp DNA stool mini kit (Qiagen) according to the manufacturer's instructions (Kato *et al.*, 2005a, b).

Typing of isolates. Typing of isolates by sequencing *slpA* was carried out with the primer set slpAcom19/slpAcom22, as described previously (Kato *et al.*, 2005a). Both strands of the amplified products were sequenced. Isolates were assigned to different major types when they had 20 or more amino acid differences, and to subtypes (01, 02, 03 and 04) when they had fewer than 20 such differences. PCR ribotyping of isolates was performed using the modified methods described by Stubbs *et al.* (1999). Briefly, the reaction volume for PCR was scaled down to 30 µl, and 1 µl DNA extracted by the method described above was used. The thermal profile was 35 cycles comprising 95 °C for 20 s and 55 °C for 120 s, followed by incubation at 75 °C for 5 min, and the resultant PCR products were separated in 2.5 % agarose gel at a constant voltage of 125 V for 3.5 h. A new PCR ribotype was identified when a banding pattern showed two or more band differences from previously identified patterns.

Detection of *tcdB* by nested PCR of DNA from stool specimens. The *tcdB* gene was detected by nested PCR on DNA extracted from stool specimens as described previously (Kato *et al.*, 2005b).

Direct typing by sequencing *slpA* of DNA from stool specimens. Amplification of *slpA* by a nested PCR was performed on DNA extracted from stool specimens that were PCR positive for *tcdB* by the nested PCR. The primer pair used for the first PCR was slpAcom19/slpAcom22 (Table 1), which was used for the typing of isolates. The *slpA* sequences of the 17 isolates representing different *slpA* sequence types were compared, and 5 forward primers (slpAcom33, slpAy32-1, slpAxr-1, slpAyok-9 and slpAog39-3) and 2 reverse primers (slpAcom30 and slpAog39-6) were selected for the second PCR for direct typing (Table 1). The primer pair slpAcom33/slpAcom30 was selected from the *slpA* sequences of eight isolates (GenBank accession nos AB180242, AB258978, AB258979, AB236153, AB249984, AB231583, AB236725 and AB236726); slpAy32-1 was from those of two isolates (AB258981 and AB258983); slpAxr-1 was from two isolates (AB239686 and AB239685); slpAyok-9 was from two isolates (AB240196 and AB257283); and slpAog39-3 and slpAog39-6 were from three isolates (AB259787, AB538230 and AF458880). The *slpA* sequences (AF458880) of ATCC 43597 (the reference strain of serogroup D) were available from the GenBank database. The thermal profiles were 35 cycles comprising 95 °C for 20 s and 55 °C for 180 s, followed by incubation at 75 °C for 5 min for the first PCR, and 35 cycles comprising 95 °C for 20 s and 55 °C for 120 s, followed by incubation at 75 °C for 5 min for the second PCR. After the first PCR with the primer pair slpAcom19/slpAcom22, a second PCR was performed by the primer pair slpAcom33/slpAcom30, which was designated primer set A. When no amplification was produced by the PCR with primer set A, the second PCR was performed separately by primer set B, consisting of primers slpAy32-1, slpAxr-1, slpAyok-9 and slpAcom30, and set C, consisting of primers slpAog39-3 and slpAog39-6. The PCR product was purified and sequenced with the same primers used for the second PCR in the same manner described for the *slpA* sequence typing on DNA extracted from isolates. Both strands of the amplified products were sequenced.

RESULTS

Typing of the reference strains

Typing analysis was performed on some epidemic strains that have been reported from around the world. ATCC 43598 strain, which was previously characterized as toxin A negative and toxin B positive (A^-B^+) and as serogroup F/PCR ribotype 017 (Delmee *et al.*, 1986; Stubbs *et al.*, 1999), was typed as *slpA* sequence type fr-01 (GenBank accession no. AB236153). The US36 strain (REA type J/PCR ribotype 001) (Killgore *et al.*, 2008) and ATCC 43599 strain had the same *slpA* sequences of type gr-01 (GenBank accession no. AB249984); NL8 strain (REA type Y/PCR ribotype 014) (Killgore *et al.*, 2008) and ATCC 43600 strain were identical by both *slpA* sequence typing (type hr-01, GenBank accession no. AB231583) and PCR ribotyping. The *slpA* sequences of the US37 strain (REA type G/PCR ribotype 002), US42 strain (REA type BI/PFGE type NAP1/PCR ribotype 027) (Killgore *et al.*, 2008) and UMCG12(3) strain (PCR ribotype 078) (Goorhuis *et al.*, 2008) were

Table 1. Primers for *slpA* sequence typing

Primer	Direction	Sequence (5′→3′)	Nt position of primer for 2nd PCR (GenBank accession no. of *slpA* of the representative isolate)
1st PCR			
slpAcom19	Forward	GTTGGGAGGAATTTAAGRAATG	
slpAcom22	Reverse	GCWGTYTCTATTCTATCDTYWCC	
2nd PCR			
Set A			
slpAcom33	Forward	TAGGYGATGGDRAWTAYGTWG	311–331 (AB180242)
slpAcom30	Reverse	CATAWBBYTYAGCTAAAKHTTBWGC	832–856 (AB180242)
Set B			
slpAy32-1	Forward	TAGGYGATGGAAAATAYGTTC	287–307 (AB258981)
slpAxr-1	Forward	TAGGTGATGGAGATTTAGTATC	299–320 (AB239686)
slpAyok-9	Forward	GTTGCAGATGGTGTAACAGGC	157–177 (AB240196)
slpAcom30	Reverse	CATAWBBYTYAGCTAAAKHTTBWGC	853–877 (AB258981)
Set C			
slpAog39-3	Forward	GTTGYWRATRHARAKTATGTTG	364–385 (AB259787)
slpAog39-6	Reverse	TTAWWSCATCAKARTCWGTTGC	955–976 (AB259787)

examined and registered as type yok-01 (GenBank accession no. AB240196), type gc8-01 (GenBank accession no. AB249986) and type 078-01 (GenBank accession no. AB470267), respectively.

Typing analysis of recovered isolates

A total of 160 stool specimens was examined and 87 *C. difficile* isolates were recovered. Of the 87 isolates, 75 were A^+B^+ and 12 were A^-B^+. Three of the A^+B^+ isolates were positive for PCR detecting the binary toxin gene ($A^+B^+CDT^+$). By *slpA* sequence typing, the 87 isolates were typed into 14 major types and further divided into 18 subtypes (Table 2). In one isolate, DJNS 0403, the *slpA* gene could not be amplified by the primer set slpAcom19/slpAcom22, but was sequenced using slpAcom19 and a reverse primer (5′-GCTGTTTGTATTCTGTCATCACC-3′). This isolate was typed as *slpA* sequence type ar-02 (Table 2). Among the 87 isolates, 18 different PCR ribotypes were identified (Fig. 1). Typing results by *slpA* sequence typing were found to be concordant with those by PCR ribotyping (Table 2). A total of 51 isolates belonging to PCR ribotype smz was classified into three *slpA* sequence subtypes (smz-01, smz-02 and smz-04), and the sequences of subtypes smz-02 and smz-04 differed by one and two deduced amino acids from that of smz-01. Two isolates showed the same PCR ribotype pattern (PCR ribotype og39), but their *slpA* amino acid sequences shared only 23 % homology. All of the 12 A^-B^+ isolates examined in the present study had the same *slpA* gene (type fr-01) but were typed into three different PCR ribotypes, 017, trf and sgf.

Direct typing by sequencing of *slpA*

A nested PCR detecting *tcdB* was performed on DNA extracted from the 160 stool specimens, and 109 (68 %) were found to be positive for *tcdB* by the nested PCR, and *tcdB*-positive *C. difficile* was detected in 86 (79 %) of the 109 stool specimens by culture (Table 3). No specimens negative for PCR detecting *tcdB* but positive for toxic culture were found. The 109 specimens that were PCR positive for *tcdB* were examined by a PCR detecting *slpA*. *slpA* was amplified by a nested PCR and sequenced in 90 (83 %) of the 109 stool specimens tested; of these 90 specimens, *slpA* typing results were obtained in 83 and 7 specimens by primer sets A and B, respectively (Tables 2 and 3). Of the 90 stool specimens in which direct typing results were obtained, 77 specimens were positive for *C. difficile* culture, and typing results from isolated strains agreed with those from direct typing in 76 specimens. In one stool specimen, the typing result on the recovered isolate (*slpA* sequence type og39-01) differed from that by direct typing with primer set A (*slpA* sequence type smz-01). *C. difficile* culture was repeated in the stool specimen, and ten colonies were selected randomly and tested. Of the ten colonies examined for PCR ribotyping, eight and two were typed as PCR ribotype smz and og39, respectively. The *slpA* genes of two of the eight smz isolates and both og39 isolates obtained were sequenced, and PCR ribotype smz and og39 isolates were typed as *slpA* sequence types smz-01 and og39-01, respectively. Second PCRs with primer sets B and C were performed on the PCR product from the first PCR from this stool specimen; *slpA* sequence type og39-01 was obtained by PCR with primer set C. In 13 stool specimens, *C. difficile* was not cultured, but typing results were obtained by direct *slpA* sequence typing (Table 3). Of the 13 stool specimens, 6 were collected after vancomycin treatment had started. No amplification product was produced by PCRs for *slpA* in nine stool specimens that were positive for *C. difficile* culture.

Table 2. Typing results of recovered isolates and direct typing results obtained from 160 stool specimens

slpA sequence type*	GenBank accession no.	PCR ribotype	Toxin production	No. of isolates/no. of stool specimens typed directly						Second primer set for direct typing
				Recovered at hospital:				Other (13†)	Total (160†)	
				A (36†)	B (44†)	C (26†)	D (41†)			
ar-02	AB259785	km0403	$A^+B^+CDT^+$	1/0					1/0	NA‡
cr-02	AB258979	km0429	$A^+B^+CDT^-$	1/1					1/1	A
cr-03	AB258980	g9376	$A^+B^+CDT^+$				1/1		1 /1	A
fr-01	AB236153	017§	$A^-B^+CDT^-$					1/1	1/1	A
		trf	$A^-B^+CDT^-$				8/9	2/3	10/12	A
		sgf	$A^-B^+CDT^-$					1/1	1/1	A
gr-01	AB249984	001§	$A^+B^+CDT^-$			2/1	2/2		4/3	A
hr-01	AB231583	014§	$A^+B^+CDT^-$		1/1		3/1		4/2	A
		gc0637	$A^+B^+CDT^-$				2/1		2/1	A
kr-02	AB258982	nt0442	$A^+B^+CDT^-$					1/1	1/1	B
xr-03	AB261625	gc0577	$A^+B^+CDT^-$				1/1		1/1	B
smz-01	AB180242	smz	$A^+B^+CDT^-$	13/14	8/12		9/9‖	1/1	31/36‖	A
smz-02	AB181350	smz	$A^+B^+CDT^-$	9 /13	5/5	1/1			15/19	A
smz-04	AB256018	smz	$A^+B^+CDT^-$			5/5			5/5	A
yok-01	AB240196	002§	$A^+B^+CDT^-$		1/0			2/2	3/2	B
yok-02	AB257283	tk0437	$A^+B^+CDT^-$		1/1				1/1	B
og39-01	AB259787	og39	$A^+B^+CDT^-$				1/1‖		1/1‖	C
t25-01	AB259786	og39	$A^+B^+CDT^-$		1/0				1/0	NA‡
gc11-01	AB258983	gc0578	$A^+B^+CDT^-$				1/1		1/1	B
gc13-01	AB269265	gc0636	$A^+B^+CDT^-$				1/1		1/1	B
gc8-01	AB249986	027§	$A^+B^+CDT^+$				1/1		1/1	A
Total no. isolates/ total no. specimens typed				24/28	17/19	8/7	30/27‖	8/9	87/90‖	

*Isolates were assigned to different *slpA* sequence major types when they had 20 or more amino acid differences, and to subtypes (01, 02, 03 and 04) when they had fewer than 20 such differences.

†No. of stool specimens examined.

‡NA, No PCR products were amplified by any of the second primer sets used.

§The nomenclature of Stubbs *et al.* (1999) was used for PCR ribotypes 017, 001, 014, 002 and 027.

‖In one specimen, two *slpA* sequence types, smz-01 and og39-01, were identified.

Endemic and sporadic types at hospitals in Japan

slpA sequence type smz (subtypes smz-01, smz-02 and smz-04)/PCR ribotype smz isolates were identified in 51 (59 %) of 86 stool specimens from which *tcdB*-positive *C. difficile* was cultured, and *slpA* of type smz was detected in 60 (67 %) of 90 stool specimens in which direct typing results could be obtained. In total, *slpA* sequence type smz was detected by culture and/or direct typing in 61 (62 %) of 99 stool specimens positive for toxic culture and/or direct *slpA* sequence typing. Type smz was found most frequently at each of the four hospitals examined here; two subtypes (smz-01 and smz-02) were predominant at hospitals A and B, and only smz-01 was found at hospital D. *slpA* sequence subtype smz-04 was found to be unique to hospital C. In the present study, eight patients suffered from *C. difficile* infection (CDI) caused by A^-B^+ isolates at hospital D, which were all typed into *slpA* sequence type fr-01/PCR ribotype trf. Among the A^-B^+ isolates examined in this study, one isolate of PCR ribotype 017 and one isolate of PCR ribotype sgf were recovered from patients admitted to the same hospital in 2004 and 2007, respectively. Both patients suffered from CDI with severe complications; the patient with the PCR ribotype 017 isolate died of CDI and the patient with PCR ribotype sgf survived after emergency colectomy. *slpA* sequence types gr, hr and yok were recovered from sporadic cases but were not predominant at any of the hospitals examined in this study. One $A^+B^+CDT^+$ isolate was found to be *slpA* sequence type gc8-01/PCR ribotype 027 in this study (Kato *et al.*, 2007). No isolates with the *slpA* gene identical to that of PCR ribotype 078 (Goorhuis *et al.*, 2008) were found among the 87 isolates tested.

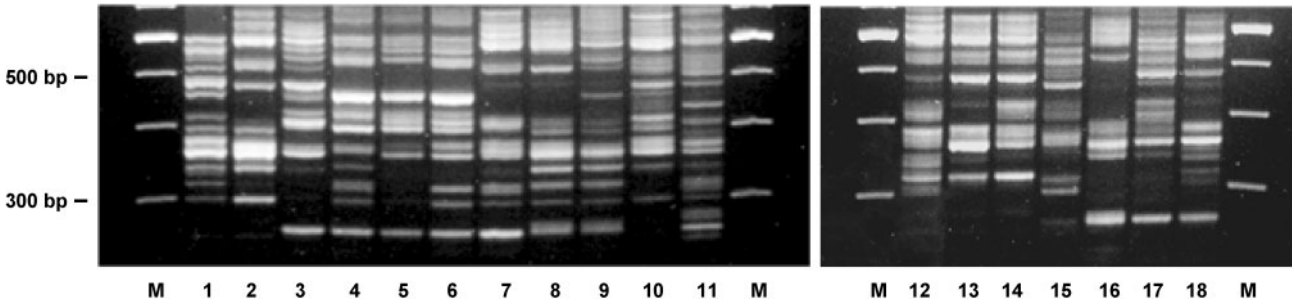

Fig. 1. PCR ribotype patterns of *C. difficile* isolates representing 18 different ribotypes. Lanes: 1, PCR ribotype km0403; 2, km0429; 3, g9376; 4, 017; 5, trf; 6, sgf; 7, 001; 8, 014; 9, gc0637; 10, nt0442; 11, gc0577; 12, smz; 13, 002; 14, tk0437; 15, 027; 16, gc0578; 17, gc0636; 18, og39; M, 100 bp ladder.

DISCUSSION

slpA sequence typing was found to have reliable typability and discriminatory power in comparison with PCR ribotyping. To date, 24 different *slpA* sequence major types and 56 subtypes have been found among *tcdB*-positive isolates (data not shown). Typing results by *slpA* sequence typing exhibited a high concordance with those by PCR ribotyping with the exception of two isolates, which belonged to the same PCR ribotype but were classified as different major types, suggesting a high variability of the *slpA* gene in this PCR ribotype. However, the limitations of this study include the low number of strains examined for each *slpA* sequence type and PCR ribotype except for types smz and fr, so further analysis using more clinical isolates is warranted to compare the typing results of the two systems.

A definite advantage of sequence-based typing techniques is the transportability of typing results from laboratory to laboratory. When an increase in the incidence of CDI or in the severity of CDI cases is perceived, or one type is found to spread to multiple patients in a healthcare facility or an area, it is prudent to ascertain whether the epidemic or endemic strain corresponds to one of the hypervirulent strains already reported worldwide (Barbut *et al.*, 2007; Goorhuis *et al.*, 2008; McDonald *et al.*, 2005; Warny *et al.*, 2005). Especially in areas or countries where there is no experience of isolating the above-mentioned strains, identification of specific strains by typing systems, which depend on banding pattern analysis such as PCR ribotyping or PFGE analysis, is difficult without obtaining previously characterized reference strains. However, if the sequence results have been registered previously in the database, obtaining reference strains for comparison is unnecessary. Although *slpA* sequence typing requires sequencing steps, once the typing results are obtained, it is easier and more objective to compare typing results of tested strains with those typed previously without repeating experiments. On this point, typing by sequencing analysis is not always time-consuming.

Table 3. Comparison of results of *tcdB*$^+$ *C. difficile* culture and direct PCR for the detection of *tcdB* and *slpA* in 160 stool specimens

tcdB$^+$ *C. difficile* culture	Direct PCR for:		No. of stool specimens (n=160)
	tcdB	*slpA*	
+	+	+	77
+	+	−	9
−	+	+	13
−	+	−	10
−	−	ND	51

ND, Not done.

slpA sequence typing was applied successfully to direct typing. Of 13 stool specimens from which *C. difficile* was not cultured but typing results were obtained by direct *slpA* sequence typing, six were collected after vancomycin treatment had started; vancomycin in the stool specimens might inhibit the growth of *C. difficile*. *slpA* sequence typing could be used to analyse *C. difficile* in some stool specimens that are inadequate for culture, such as those obtained after starting treatment with vancomycin or metronidazole. Direct typing by sequencing of *slpA* at present has the limitation of the tedious steps required. As this typing method depends on the variability of the *slpA* gene, three sets of primers for the second PCR are needed to amplify the variable region of the gene, which complicates the procedure. Although the procedure must be simplified for practical use, the method could be valuable for detecting epidemiologically important strains.

Toxin B-positive *C. difficile* was recovered from 86 (54 %) of 160 stool specimens tested. The stool specimens tested in this study were obtained from patients who had symptoms that were considered severe and so the samples were sent to the National Institute of Infectious Diseases for further analysis. This is the major reason for the high prevalence.

The type smz strain has been documented to cause healthcare-associated infection in many hospitals in Japan

(Kato *et al.*, 2001, 2005a; Sawabe *et al.*, 2007). Killgore *et al.* (2008) tested 42 *C. difficile* isolates from four countries in North America and Europe using seven techniques including *slpA* sequence typing, and none was identified as *slpA* sequence type smz. In another report, only 3 of 33 isolates recovered from an outbreak in the USA were type smz (Kato *et al.*, 2001, 2005a). Joost *et al.* (2009) examined the *slpA* sequences of their *C. difficile* isolates and compared the sequence results with those registered in the database; they found type smz in only 3 % of isolates recovered from CDI patients at a university hospital in German. Whilst these limited studies suggest that type smz is not frequently isolated in North America or Europe, the real prevalence is unknown. *slpA* sequence typing could be useful for studying the distribution of the smz strain worldwide.

A^-B^+ *C. difficile* has been reported to cause nosocomial outbreaks (Komatsu *et al.*, 2003; Kuijper *et al.*, 2001) and to be predominant in Ireland, Poland (Barbut *et al.*, 2007; Pituch *et al.*, 2007) and Korea (Kim *et al.*, 2008), as well as in Japan (Rupnik *et al.*, 2003), indicating the emergence of CDI caused by A^-B^+ *C. difficile*. As far as we know, A^-B^+ isolates that have 1.8 kbp deletions in the repeating sequences of *tcdA* (Kato *et al.*, 1999), including the ATCC 43598 strain, are all typed into the *slpA* sequence major type fr together with exceptional isolates that belonged to serogroup X (data not shown). Notably, A^-B^+ *C. difficile* was isolated from two patients representing two sporadic cases with severe complications, although no specific characteristics were found in these isolates. In the present study, 1 isolate was identified as PCR ribotype 027; none of the 87 isolates tested was PCR ribotype 078. To date, we have found only sporadic CDI cases caused by *slpA* sequence type gc8/PCR ribotype 027 isolates in Japan, which were historic isolates (Kato *et al.*, 2007; Sawabe *et al.*, 2007). It has been documented that PCR ribotype 078 more frequently causes community-associated diseases (Goorhuis *et al.*, 2008). All patients examined in this study had healthcare-associated infection, which might be one of the reasons why CDI caused by PCR ribotype 078 was not found. Typing by sequencing of *slpA* could be a reliable tool for discovering CDI cases and outbreaks due to these epidemic strains, which may have been overlooked.

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