BACTERIAL EPIDEMIOLOGY AND TYPING

Colonisation and transmission of *Clostridium difficile* in healthy individuals examined by PCR ribotyping and pulsed-field gel electrophoresis

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Healthy adults who had not been exposed to antimicrobial agents for the preceding 4 weeks were examined for intestinal carriage of *Clostridium difficile*. The 1234 individuals examined were composed of seven groups: three classes of university students, hospital workers at two hospitals, employees of a company and self-defence force personnel at a local station. Overall, 94 (7.6%) individuals were positive for *C. difficile* by faecal culture but carriage rates among the study groups ranged from 4.2% to 15.3%. Typing by PCR ribotyping and pulsed-field gel electrophoresis demonstrated clusters of carriers colonised by a single type in each of three groups, indicating that cross-transmission of *C. difficile* can occur in community settings. Follow-up culture was performed on 38 *C. difficile*-positive individuals and *C. difficile* was isolated again from 12 (32%) of them 5–7 months after the initial culture; six (50%) of these 12 individuals had a new strain on repeat culture. Two or more family members were *C. difficile*-positive in five of 22 families examined. *C. difficile* with an identical type was isolated from persons within a family in only one family. These results suggest that intestinal carriage by healthy adults may play a role as a reservoir for community-acquired *C. difficile*-associated diarrhoea, but that cross-transmission of *C. difficile* does not occur frequently among family members at home.

Introduction

Clostridium difficile is the principal pathogen causing antibiotic-associated diarrhoea and colitis. It has been well documented that this organism spreads nosocomially and causes hospital outbreaks of *C. difficile*-associated diarrhoea (CDAD) in various clinical settings [1, 2]. In contrast, less information is available about community-acquired CDAD. In one report, the prevalence of community-acquired CDAD was only 7.7 cases per 100 000 person-years [3], but others have reported that community-acquired CDAD is unexpectedly common [4] and that *C. difficile* might be a significant cause of community-acquired diarrhoea [5]. Little attention has been paid to the routes of

transmission and potential reservoirs of *C. difficile* in the community. In the hospital setting, infected and colonised patients and contaminated inanimate objects have been implicated as potential sources of *C. difficile*. It is uncertain whether intestinal carriage by healthy individuals is an important reservoir and an exogenous source for community-acquired CDAD.

In the present study, 1234 healthy adults in seven different study groups were examined for *C. difficile* carriage. Two typing systems, PCR amplification of rRNA intergenic spacer regions (PCR ribotyping) and pulsed-field gel electrophoresis (PFGE), were used to analyse the isolates from carriers to determine whether transmission of *C. difficile* among healthy individuals in the community occurs frequently. Family contacts of *C. difficile* carriers were also examined to investigate how frequently transmission of *C. difficile* can occur among family members.

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Materials and methods

Study population

A total of 1234 healthy adults (age range 18–65 years, median 27 years) was examined for intestinal carriage of *C. difficile* by stool culture; none had been exposed to antimicrobial agents for at least 4 weeks before the beginning of the study. Three study groups, A, B and C, consisting of 36, 100 and 98 individuals, respectively, were classes of university students; two groups, D and E, comprised 190 and 94 hospital workers at two hospitals; group F was 89 employees of a company; and group G consisted of 627 ground self-defence force personnel (Table 1). A follow-up stool culture was requested after 5–7 months from the individuals whose cultures were positive for *C. difficile*. Family members of *C. difficile*-positive individuals were examined for *C. difficile* carriage.

Isolation and identification

Stool specimens were treated with alcohol for spore selection and cultured on cycloserine-cefoxitin-mannitol agar (CCMA) for isolation of *C. difficile*. The composition of CCMA was (/L): Proteose Peptone No. 2 (Becton Dickinson, Sparks, MD, USA) 40 g; agar 20 g; Na₂HPO₄ 5 g; KH₂PO₄ 1 g; NaCl 2 g; MgSO₄ 0.1 g; mannitol 6 g; neutral red (1% in ethanol) 3 ml; sodium taurocholate (Nacalai Tesque, Kyoto, Japan) 1 g; cycloserine (Nacalai Tesque) 300 mg; cefoxitin (Sigma) 10 mg. One volume of stool was diluted with an approximately equal volume of Brain Heart Infusion Broth (Becton Dickinson) containing cysteine (Wako, Osaka, Japan) 0.05% and mixed with twice the volume of ethanol 95%. After incubation for 30 min at room temperature, 100 µl of the sample mixture were inoculated on to prereduced CCMA and incubated at 37°C for 48 h anaerobically in anaerobic jars filled with a gas mixture containing N₂ 80%, CO₂ 10% and H₂ 10%. To investigate potential carriage of multiple strains by individuals, a maximum of five colonies was randomly selected from the primary culture plate for each specimen for separate subculture and examination. *C. difficile* was identified as described previously [6]. Toxigenicity of the isolates was determined by PCR assay. Two primer sets were used to detect the toxin A gene; primers NK3 and NK2 were derived from the non-repeating portion of the *C. difficile* toxin A gene, and primers NK11 and NK9 were derived from the repeating portion of the *C. difficile* toxin A gene [6, 7]. A segment of the toxin B gene was amplified by primer set NK104-NK105, which were derived from the non-repeating sequences of the toxin B gene [6]. The strains which were positive by PCR with NK3-NK2 and generated a PCR product of c. 1200 bp by PCR with primer set NK11-NK9 were identified as toxin A-positive, toxin B-positive (A+, B+). The strains which were positive by PCR with NK3-NK2 and yielded a PCR product of c. 700 bp by PCR with primer set NK11-NK9 were identified as toxin A-negative, toxin

Table 1. Asymptomatic intestinal colonisation by *C. difficile* in healthy adults in seven study groups and toxigenicity and typing results of isolates from each group

Study group*	Age (years)		Number of individuals tested	Number (%) of individuals positive for <i>C. difficile</i>	Number of isolates of toxin-producing type			Number of types identified by		
	Range	Median			A+, B+	A-, B+	A-, B-	PCR ribotyping	PFGE Major type	Subtype
A University student	20–22	20	36	3 (8.3)	3	0	0	3	3	3
B University student	20–38	22	100	7 (7.0) [†]	2	0	6	7	6 [‡]	6
C University student	20–30	21	98	15 (15.3)	13	0	2	9	8 [§]	10
D Hospital worker	21–58	28	190	8 (4.2)	2	1	5	8	8	8
E Hospital worker	19–56	34	94	4 (4.3)	1	3	0	2	2	2
F Company employee	20–65	45	89	4 (4.5)	3	0	1	4	4	4
G Self defence force personnel	18–55	31	627	53 (8.5)	27	0	26	24	32	41
Total	18–65	27	1234	94 (7.6)	51	4	40	37	49	66

*Study groups A, B and C were different classes of a university, and D and E were hospital workers at two different hospitals.

[†]Isolates with two kinds of toxin-producing types (A+, B+ and A-, B-) were obtained from one specimen.

[‡]Six of eight isolates were resolved into six major types and subtypes by PFGE; two isolates were non-typable because of DNA degradation.

[§]Fourteen of 15 isolates were resolved into eight major types and 10 subtypes by PFGE; one isolate was non-typable.

^{||}Fifty-two of 53 isolates were resolved into 32 major and 41 subtypes by PFGE; one isolate was non-typable.

B-positive (A–, B+). Toxin A-negative, toxin B-negative (A–, B–) strains were determined by negative results in PCR with primers NK3–NK2. Presence of the toxin B gene was confirmed by PCR with primers NK104–NK105.

Typing

PCR ribotyping was performed by the method described by Stubbs *et al.* [8] with some modification. Briefly, the reaction volume for PCR was scaled down to 50 µl and PCR products were concentrated by heating at 75°C for 30 min and separated by electrophoresis in agarose (Nacalai Tesque) 3% at a constant voltage of 120 V for 4 h. Isolates with patterns that differed by one or more major band were assigned to different PCR ribotypes; differences in faint bands were ignored. PFGE analysis was performed as described previously [9]. DNA in the inserts was digested with *Sma*I (New England Biolabs, Beverly, MA, USA) and resulting macrorestriction fragments were resolved by PFGE at a constant voltage of 6 V/cm with 25-s pulses for 3 h followed by 50-s pulses for 20 h. Major PFGE types were defined by more than three fragment differences, and these major types were subtyped by three or fewer than three fragment differences according to the criteria described by Tenover *et al.* [10].

Results

Asymptomatic colonisation and transmission in healthy adults

C. difficile was isolated from 94 (7.6%) of the 1234 asymptomatic individuals examined. The carrier rates ranged from 4.2% to 15.3% among the seven study groups (Table 1). Identification of toxigenicity and PCR ribotyping were performed on all isolates representing a maximum of five colonies from each specimen. All isolates from a specimen gave an identical PCR ribotype profile in 93 specimens (98.9%). One specimen yielded isolates with two different PCR ribotypes, and these two isolates also differed in toxin-producing types. One isolate from each of the 93 specimens and two isolates of different PCR ribotypes from the one specimen were analysed by PFGE. Table 1 shows the carriage rate, toxigenicity and typing results of isolates from each study group. Overall, 51 (53.7%) of the 95 isolates were A+, B+, four (4.2%) were A–, B+, and 40 (42.1%) were A–, B–.

All 95 isolates were typable by PCR ribotyping and were resolved into 37 ribotypes. Four isolates were non-typable by PFGE because of DNA degradation during sample processing. The 91 PFGE-typeable isolates were classified into 49 major types and 66 subtypes. Among the four isolates non-typable by PFGE, three PCR ribotypes were identified. All isolates within the same PFGE major type showed the same

banding pattern by PCR ribotyping. PCR ribotype hr was isolated most frequently and accounted for 17 (17.9%) of the 95 isolates. These 17 PCR ribotype hr isolates were classified into five major types and further resolved into nine subtypes by PFGE. No isolates of PCR ribotype smz, which was found most frequently from CDAD patients in Japanese hospitals (unpublished observations), were found in the healthy carriers.

Clusters of individuals who were carriers of the same PFGE type were found in groups C, E and G (Table 2). Of 15 isolates from student group C, five isolates were identical according to PFGE (subtype Hr-a) (Table 2 and Fig. 1, lanes 8–12). Follow-up culture after 6 months on four of these five individuals showed that one still carried *C. difficile* of the same type and two were colonised by isolates of a type different from type Hr-a and from each other; *C. difficile* was not detected from the remaining individual. Isolates identified as PFGE major type Hr were also found in three individuals of study group G. In worker group E, four of 94 individuals were positive for *C. difficile* and three of them harboured isolates with an identical PFGE pattern (subtype Fr-b). When re-examined after 6 months, one still carried the type Fr-b strain, one had an isolate of a different type and the third was negative for *C. difficile*. Isolates with the same PFGE pattern (type Z206) accounted for five of 53 isolates from individuals in group G. Specimens from only two of the five individuals with type Z206 were obtained for follow-up culture; one individual continued to have the same type Z206 strain and the other was culture-negative. Ten of the 53 isolates from group G were assigned to the same PCR ribotype (sbt) and PFGE major type (NK201) and were further resolved into five PFGE subtypes by three or fewer band differences (subtypes NK201-a–e). Two of the 10 individuals were followed up; one carried *C. difficile* of the same type as the initial isolate and the other was *C. difficile*-negative.

Two isolates from study group C that showed the same PCR ribotype differed by one band on PFGE (subtypes M239-a and -b) (Fig. 1, lanes 1 and 2 and Table 2). Similarly, another pair of isolates from group C belonging to the same PCR ribotype gave different PFGE subtypes by three band differences (subtypes M246-a and -b) (Fig. 1, lanes 3 and 4). No isolates of these two PCR ribotypes were obtained from individuals in groups other than C. Similar findings were observed with four pairs of isolates from group G. Follow-up culture was not performed on these 12 individuals.

In all, 38 of 94 *C. difficile*-positive individuals were re-examined 5–7 months later. *C. difficile* was isolated again from 12 individuals (31.6%). Typing by PCR ribotyping and PFGE showed that six (50%) of the 12 individuals yielded isolates of the same type as their initial strain and five (42%) harboured a new strain

Table 2. Carriage of *C. difficile* isolates identified as the same PFGE major type by more than two individuals within each study group

PFGE		Toxin-producing type	Number of individuals from study groups						
Major type	Subtype		A (3)*	B (7)	C (15)	D (8)	E (4)	F (4)	G (53)
Hr	-a	A+, B+			5				2
	-b	A+, B+							1
M239	-a	A+, B+			1				
	-b	A+, B+			1				
M246	-a	A+, B+			1				
	-b	A+, B+			1				
Z206		A+, B+							5
Z78		A+, B+							2
Fr	-a	A-, B+				1			
	-b	A-, B+					3		
NK201	-a	A-, B-				1			5
	-b	A-, B-							1
	-c	A-, B-							2
	-d	A-, B-							1
	-e	A-, B-							1
Dr	-b	A-, B-							1
	-c	A-, B-							1
Z217	-a	A-, B-							1
	-b	A-, B-							1
Z612	-a	A-, B-							1
	-b	A-, B-							1
Z766	-a	A-, B-							1
	-b	A-, B-							1
Other types			3	5	5	6	1	4	24
Non-typable†				2	1				1

*Number of individuals who were positive for *C. difficile*.

†Non-typable by PFGE analysis because of DNA degradation.

Fig. 1. PCR ribotype patterns (a) and PFGE patterns (b) of *C. difficile* isolates from 15 individuals of study group C. Lanes M, standard 100-bp DNA ladders (a) and chromosomal DNA from *Saccharomyces cerevisiae* (b) as mol. wt standards; lanes 1–15, isolates from 15 individuals of group C. The isolate that represents the PCR ribotype pattern in lane 15 of panel a was non-typable by PFGE because of DNA degradation and is not shown in panel b.

represented by all isolates (one-to-five colonies) from each specimen on repeat culture. One individual harboured isolates of both a new type and the same type as the initial strain; four of five colonies examined

represented a new type and one colony was identified as the same type as the initial strain. Overall, seven (18.4%) of the 38 individuals re-examined carried isolates identified as the same type before and after a

half-year period. There was no correlation between colonisation state and typing results.

Colonisation and transmission in family members

To determine how frequently transmission of *C. difficile* occurs among family members, 22 families were examined; the 22 index persons who had been found to carry *C. difficile* at initial examination and 56 of their family contacts were tested for *C. difficile* carriage. All 30 family members, including 10 index persons re-examined in this part of the study, from 10 of the 22 families were negative for *C. difficile*. In one family, *C. difficile* was isolated from one family contact (a 3-year-old child) while the index person was negative at the repeated culture. In six families comprising 25 persons, only the index person of each family harboured *C. difficile*. *C. difficile* was detected from two or more members within each of the remaining five families. The results of *C. difficile* isolation and typing of isolates from the five families are shown in Table 3. Three of these five families were re-examined 6–7 months after the initial culture was done (families 1–3). In one family, three of five members, including the index person, were positive for *C. difficile* at both the initial and repeated examination (Table 3, family 1-1 and family 1-2). *C. difficile* was not detected in the repeated culture from any of the

members of the other two families (families 2 and 3). In four of the five families, different PCR ribotyping and PFGE profiles were obtained with isolates from members within each family (families 1, 3, 4 and 5). Three members of family 1 harboured isolates different from each other at both the initial and the repeated cultures (Fig. 2, lanes 1–6). In family 2, *C. difficile* isolates from the index person and her 1-year-old son were the same type by both PCR ribotyping and PFGE (Fig. 2, lanes 7 and 8).

In one member of family 1 (sister-a), although isolates of the same PCR ribotype (ysg) were obtained from both initial and repeated culture, the two isolates were of different PFGE major types because they had four band differences (Fig. 2, lanes 2 and 5). In other members of family 1, in contrast, the index person had acquired a new strain at follow-up examination (Fig. 2, lanes 1 and 4), and another member (sister-b) continued to have a strain of the same PCR ribotype and PFGE type as that from her initial culture (Fig. 2, lanes 3 and 6).

Discussion

In previous reports of intestinal carriage of *C. difficile* in healthy adults, carriage rates ranged from 0% to

Table 3. Culture and typing results of *C. difficile* in families in which two or more members were *C. difficile* carriers

Family	Member	Gender	Age (years)	Date of isolation (m/y)	<i>C. difficile</i> isolation*	Toxigenicity of isolate	Typing result	
							PCR ribotype	PFGE type
1-1†	Father	Male	47	11/1998	–			
	Mother	Female	45	11/1998	–			
	Index person	Male	20	11/1998	+	A–, B–	gns	NT‡
	Sister-a	Female	18	11/1998	+	A+, B+	ysg	MF353
	Sister-b	Female	14	11/1998	+	A+, B+	yok	OG02
1-2	Father	Male	47	7/1999	–			
	Mother	Female	45	7/1999	–			
	Index person	Male	20	7/1999	+	A–, B–	m986	M986
	Sister-a	Female	18	7/1999	+	A+, B+	ysg	MF989
	Sister-b	Female	14	7/1999	+	A+, B+	yok	OG02
2	Husband	Male	30	6/1998	–			
	Index person	Female	27	6/1998	+	A+, B+	hr	Hr-a
	Daughter	Female	3	6/1998	–			
	Son	Male	1	6/1998	+	A+, B+	hr	Hr-a
3	Index person	Female	32	11/1998	+‡	A+, B+	hr	Hr-b
	Daughter	Female	3	11/1998	+	A–, B–	dr	KN167
	Son	Male	1	11/1998	+	A–, B–	xr	KF391
4	Father	Male	63	4/1999	–			
	Mother	Female	58	4/1999	+	A+, B+	yok	NF582
	Index person	Female	33	4/1999	+	A–, B–	mre	NT
5	Father	Male	55	12/1999	–			
	Mother	Female	51	12/1999	–			
	Brother	Male	24	12/1999	–			
	Index person	Male	22	12/1999	+	A+, B+	hr	KN159-c
	Sister	Female	19	12/1999	+	A–, B–	sgw	MF380

NT, non-typable.

* –, negative; +, positive.

†Families 1-1 and 1-2 were the same family tested after a 7-month interval.

‡Two different types of *C. difficile* were isolated from the specimen.

Fig. 2. PCR ribotype patterns (a) and PFGE patterns (b) of *C. difficile* isolates from members of two families. Lanes M, standard 100-bp DNA ladders (a) and chromosomal DNA from *Saccharomyces cerevisiae* (b) as mol. wt standards; lanes 1 and 4, isolates from the index person of family 1; lanes 2 and 5, isolates from sister-a in family 1; 3 and 6, isolates from sister-b in family 1; 7, an isolate from the index person of family 2; 8, an isolate from the 1-year-old son of the index person of family 2. The isolates of lanes 4, 5 and 6 were cultured (family 1-2) 7 months after the culture of isolates of lanes 1, 2 and 3 (family 1-1).

17.5%, with the highest rates reported in Japan [11–15]. These results may have been influenced by many factors, such as size of the study, age of the population, procedures for sample collection and culture methods. Among the population groups studied here, carriage rates ranged from 4.2% to 15.3%. The difference in carriage rates in this study indicates the influences of factors other than culture procedures or ethnic origin. There was no evidence that healthcare workers carry *C. difficile* at higher rates than do individuals with other occupations, supporting the idea that intestinal carriage in healthcare workers is not a major concern with regard to their possibly being a reservoir of *C. difficile* in hospitals [16].

In 93 (98.9%) of 94 *C. difficile* carriers, all the isolates from the same specimen belonged to the same types according to PCR ribotyping, suggesting homogeneity of intestinal *C. difficile* strains in healthy individuals. The PCR ribotype strain identified as the endemic

strain causing multiple outbreaks in Japanese hospitals (unpublished observations) was not found in any of the healthy carriers, indicating that the hospital endemic strain is, at least, not common among Japanese people and may have specific potential for nosocomial infection.

In the present study, typing results demonstrated healthy carriers in clusters colonised by a single type in some study groups. This suggests that cross-transmission of *C. difficile* can occur not only in nosocomial settings but also among healthy adults in community settings. One of the factors that might influence the carriage rate could be the state of cross-transmission of *C. difficile* at the time of sampling. Transmission routes of *C. difficile* among individuals who are neither hospitalised patients nor nursing home residents are unknown; however, nosocomial transmission of *C. difficile* among hospitalised patients has been well documented [1, 2, 9]. A reduction in CDAD rate associated with the use of vinyl gloves indicated that hand carriage by healthcare workers contributes to transmission [17]. Another investigation found that the incidence of CDAD was reduced by introducing tympanic thermometers in place of disposable oral and rectal thermometers [18], suggesting that cross-transmission may occur during temperature taking, especially by the rectal route. However, healthy adults who study or work at the same place may have little chance to be in close proximity to each other. Although there are no data about contamination of the environment surrounding the individuals examined in the present study, healthy carriers appear to contaminate the environment much less than hospitalised patients with diarrhoea. Even in nosocomial settings, the exact role of the contamination of environmental surfaces in transmission of *C. difficile* is controversial, although it has been well reported that the environmental surfaces of hospitals can be contaminated with *C. difficile* [2, 19, 20]. In other words, evidence of *C. difficile* transmission among healthy individuals indicates that nosocomial transmission may occur even in the absence of any close contact with objects heavily contaminated with *C. difficile*.

It should be noted that transmission of *C. difficile* among family members was not commonly found, although the number of families tested here was limited. A *C. difficile* isolate of the same type resulting from family contact was found in only one of 11 families in which at least one adult individual was colonised by *C. difficile* when examined. Data from the present study indicate that the home is not an important reservoir for *C. difficile* in the community. In contrast, Sutphen *et al.* analysed families of children with chronic diarrhoea and demonstrated that *C. difficile*-positive family contacts were common [21]. In the case of patients with intestinal symptoms, especially diarrhoea, the home environment may be heavily contaminated and may be a reservoir of *C. difficile* [20].

A follow-up culture after *c.* 6 months demonstrated that 32 (84.2%) of 38 asymptomatic carriers were negative for *C. difficile* or harboured a different strain, indicating that most intestinal carriage is not a permanent state. Notably, six (50%) of 12 individuals who were *C. difficile*-positive at the time of repeated culture continued to have an identical strain and six (50%) acquired a new strain. This result is in accord with the findings that approximately half of recurrences of CDAD in hospitalised patients were re-infections with a different strain [22, 23]. A similar finding was observed in asymptomatic carriers treated with vancomycin [24].

In this study, the guideline described by Tenover *et al.* [10] was used to interpret DNA fragment patterns generated by PFGE. According to the guideline, differences of three or fewer restriction-fragment positions could have occurred *via* a single genetic event and isolates with three or fewer differences are considered to be subtypes and closely related [10]. Of 53 isolates from individuals of study group G, 10 isolates were designated as the same PCR ribotype (sbt) and PFGE major type (NK201); these 10 isolates gave five different PFGE profiles that differed by three or fewer bands. In contrast, only one of the 42 isolates from the groups other than G was identified as ribotype sbt/PFGE major type NK201. This suggests that genetic changes occurred in the strain of type NK201 while it was passing through the gut of the individuals in group G, and such genetic events might result in minor variations of the PFGE patterns. In contrast, all five isolates of PFGE type Z206 from group G had an identical PFGE pattern, suggesting that genetic changes – at least at restriction sites recognised by the restriction endonuclease used – might be less likely to occur in the type Z206 strain than in the type NK201 strain. Similar findings to those observed with type NK201 were observed in some pairs of isolates, supporting the idea that the criteria of Tenover *et al.* [10] are applicable to *C. difficile* isolates in a community setting.

A pair of isolates from the same person obtained 7 months apart showed the same PCR ribotype pattern but the PFGE patterns differed by four bands. In this case, it is more likely that genetic changes in a strain resulted in different PFGE patterns than that a different strain of the same PCR ribotype as that of the initial strain was newly acquired during the 7-month period.

A combination of PCR ribotyping and PFGE was useful for the investigation of *C. difficile* isolates from healthy carriers. PFGE was more discriminatory and it was easier to compare the typing results than with PCR ribotyping [25], but PFGE had the disadvantage of DNA degradation in some isolates which could be analysed by PCR ribotyping [9, 25]. Furthermore, PCR ribotyping provided information on the genetic rela-

tionship between isolates that displayed different PFGE profiles.

In the present study, transmission of *C. difficile* was demonstrated among healthy individuals in a community setting, suggesting a possible role of intestinal carriage in healthy adults as a reservoir for community-acquired CDAD. Further study of *C. difficile* isolates from community-acquired CDAD in comparison with those from healthy carriers is required to elucidate how *C. difficile* is transmitted and causes intestinal diseases in the community.

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