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Coexistence of multiple PCR-ribotype strains of *Clostridium difficile* in faecal samples limits epidemiological studies

Renate J. van den Berg,[1] Hadi A. A. Ameen,[1] Takahiro Furusawa,[1] Eric C. J. Claas,[1] Eric R. van der Vorm[2] and Ed J. Kuijper[1]

[1]Department of Medical Microbiology, Centre for Infectious Diseases, Leiden University Medical Centre, Leiden, The Netherlands

[2]Department of Medical Microbiology, VU University Medical Centre, Amsterdam, The Netherlands

Correspondence
Ed J. Kuijper
e.j.kuijper@lumc.nl

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Clostridium difficile is an important cause of antibiotic-associated diarrhoea. The simultaneous presence of different strains in individual faecal samples has not yet been established, but is important for epidemiological studies. Recurrences of *Clostridium difficile*-associated diarrhoea (CDAD) are observed in 15–20 % of patients and have been reported as relapses or reinfections with a new strain. In a period of 1 year, 28 faecal samples from 23 patients with a first episode of CDAD were collected at the Leiden University Medical Centre. In addition, 52 faecal samples from 23 patients, from three different hospitals, with one ($n = 19$), two ($n = 2$) or three ($n = 2$) recurrences were studied. PCR-ribotyping was applied as the standard typing method for the isolates. The toxinogenic and clindamycin-resistance profiles of the isolates was determined by PCR. Of 23 patients with a first episode of CDAD, two (8·7 %) harboured two different types, with no differences in toxinogenicity or clindamycin resistance, within one faecal sample. One of these 23 patients showed two types in three faecal samples from the same episode. Of the 23 patients with recurrences, six (26 %) showed a different strain type isolated in a recurrent episode. The number of cases of multiple *C. difficile* strains in faecal samples from patients with a first episode of CDAD did not differ significantly from the number of different strains present in recurrent episodes (chi-square test, $P \leqslant 0{\cdot}2$). This observation limits the application of typing methods for studying the epidemiology of CDAD.

INTRODUCTION

Clostridium difficile has been recognized as a cause of nosocomial diarrhoea and pseudomembranous colitis. Its enteropathogenicity is associated with the production of enterotoxin A (308 kDa) and cytotoxin B (270 kDa) (Barroso *et al.*, 1990; Dove *et al.*, 1990). Clinical isolates from patients with nosocomial diarrhoea or pseudomembranous colitis usually produce both TcdA and TcdB, but an increasing number of reports mention severe infections and outbreaks due to TcdA-negative, TcdB-positive strains (al-Barrak *et al.*, 1999; Alfa *et al.*, 2000; Kuijper *et al.*, 2001). It has been reported previously that clindamycin resistance is high among these strains, in contrast to strains that are TcdA- and TcdB-positive (van den Berg *et al.*, 2004).

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Abbreviations: CDAD, *Clostridium difficile*-associated diarrhoea; REA, restriction enzyme analysis.

Patients often develop a recurrent *C. difficile* infection (15–20 %) after discontinuation of antibiotic therapy (Wilcox & Spencer, 1992). Recurrences can be explained by endogenous persistence of *C. difficile* spores (relapse) or by the acquisition of a new strain from an exogenous source (reinfection). Determining if a recurrence is due to a relapse or a reinfection is important for epidemiological studies of *C. difficile*. There are conflicting data from studies into the simultaneous presence of different strains in individual faecal samples using molecular typing methods and immunochemical assays (Wilcox *et al.*, 1998; O'Neill *et al.*, 1991; Devlin *et al.*, 1987; Borriello & Honour, 1983; Sharp & Poxton, 1985).

PCR-ribotyping has been described as a robust method for the genotyping of *C. difficile* strains, although restriction enzyme analysis (REA) is also frequently applied. REA is able to subgroup PCR-ribotypes (Johnson *et al.*, 2003), but is a difficult method to interpret and lacks objective interpretation (Cohen *et al.*, 2001). Stubbs *et al.* (1999) applied the PCR-ribotyping method to 2030 strains and differentiated 116 genotypes. All known serogroups could be differentiated

by this method as well (Stubbs *et al.*, 1999; van den Berg *et al.*, 2004). Therefore, this PCR-ribotyping method was used to investigate the occurrence of different *C. difficile* isolates in faecal samples from patients with one or more episodes of *C. difficile*-associated diarrhoea (CDAD).

Additionally, all isolates were characterized by PCR for the exact profile of *tcdA* and *tcdB*, and for clindamycin resistance, *erm*(B).

METHODS

Patients included in this study. In 2002, all faecal samples positive by both enzyme-linked fluorescence assay (VIDAS CDA2) and culture for *C. difficile* at the bacteriological laboratory in the Leiden University Medical Centre were stored in glycerol broth (50 % w/v) at −80 °C. A total of 28 faecal samples from 23 patients with a first episode of CDAD were available for the current study. More than one faecal sample from the same diarrhoeal episode was included for four patients (numbers 7, 12, 17 and 21, Table 1). For comparison, *C. difficile* strains from 23 patients with recurrent *C. difficile* infections were obtained from three different hospitals (Table 2). The Academic Medical Centre, Amsterdam (hospital I) provided *C. difficile* isolates from 14 patients with recurrences, collected over a period of 11 years (1989–2000). *C. difficile* strains cultured from five patients with CDAD recurrences in a period of 7 months (May–November 2003) at the VU University Medical Centre, Amsterdam (hospital II) were also available for this study. The remaining four patients with CDAD recurrences were obtained at the Leiden University Medical Centre (hospital III) from June 2002 to April 2003.

Faecal culture. Faecal samples treated with an ethanol shock pre-treatment prior to inoculation were plated onto Columbia agar containing colistin and nalidixic acid (CNA) and/or onto *C. difficile* agar with moxalactam, norfloxacin and cystein (CDMN), and were incubated in an anaerobic environment at 37 °C for 2 days (Aspinall & Hutchinson, 1992). CDMN medium was also inoculated with faecal samples not pre-treated with ethanol. Colonies of Gram-positive rods with subterminal spores were tested for the production of L-proline-aminopeptidase and for hydrolysis of aesculin (Garcia *et al.*, 1997) in hospitals I and III, whereas hospital II performed a cytotoxin assay on Vero cells for verification of toxinogenic culture. DNA was isolated from subcultures of individual colonies. A total of five colonies from each faecal sample were picked for DNA isolation: three colonies from the two culture plates after ethanol-shock treatment (CDMN or CNA plates), and two colonies from the CDMN plate inoculated with untreated faecal samples.

DNA isolation. DNA was isolated from colonies of *C. difficile* by QiaAmp DNA isolation columns (Qiagen) according to the manufacturers recommendations, including a 10 min incubation at 55 °C with proteinase K (Qiagen). The final volume of the DNA extracts was 200 μl.

PCR-ribotyping. The method described by Bidet *et al.* (1999) was used. The template DNA was amplified with the PRB primers (Table 3). The amplification reactions were performed in a 50 μl final volume containing 25 μl HotStar Taq Mastermix (Qiagen), 10 pmol of each primer and 5 μl DNA. After an initial enzyme activation step of 15 min at 95 °C, the protocol consisted of 35 cycles of 1 min at 94 °C for denaturation, 1 min at 57 °C for annealing, and 1 min at 72 °C for elongation. The amplified products were analysed by agarose gel electrophoresis. PCR-ribotyping codes for the two different patient groups were assigned as sequential numbers.

Genetic identification of *tcdA* and *tcdB* profiles. All isolates were tested for the presence of genes *tcdA* and *tcdB*. For the detection of *tcdA*, primers NKV011 and NK9 (Table 3) were used, as described by Kato *et al.* (1999). TcdA-positive strains showed a 2535 bp amplicon size. The *tcdB* profile was verified using primers NK104 and NK105 (Table 3), as described by Kato *et al.* (1998). The presence of a 204 bp fragment was considered as indicative of the presence of *tcdB*. The amplified products were analysed by separation by electrophoresis on agarose gels.

Genetic identification of clindamycin resistance. Clindamycin resistance was tested by PCR. The target was the *erm*(B) gene, which codes for macrolide-lincosamide-streptogramin (MLS) resistance, as described previously by Sutcliffe *et al.* (1996). The primers used are described in Table 3. Clindamycin-resistant strains were defined as strains with a 639 bp amplicon size. The amplified products were analysed by separation by electrophoresis on agarose gels.

RESULTS

Typing of *C. difficile* isolates from 28 faecal samples from 23 patients with a first episode of CDAD

Cultures for *C. difficile* were performed for 28 faecal samples from 23 patients with a first episode of CDAD. Of the 23 patients, 52 % were male and 48 % female, and the median age was 59·1 years (range 13–79). Of these 23 episodes, 35 % were diagnosed in outpatients, 13 % in patients at the gastroenterology department, 9 % in patients at the nephrology department and 9 % in patients at the internal medicine department. Severe cases of CDAD were seen in seven of 23 patients (30 %) and mild cases in 16 patients (70 %) (Table 1). Severe cases were defined as having bloody diarrhoea with a high fever, hypovolumia, peripheral blood leukocytosis and hypoalbuminemia or with pseudomembranous lesions by endoscopy. In a follow-up observation period of 2 years, seven patients (numbers 4, 11, 16, 17, 21, 22 and 23, Table 1) showed a recurrence of a *C. difficile* infection. Faecal samples from recurrent episodes of patients 11, 16 and 17 were available for further study and therefore these patients were included in the group with recurrent CDAD also (patients III-2, III-1 and III-4, Table 2).

For PCR-ribotyping, five isolates per sample were tested if possible, but from five faecal samples only four ($n = 3$), three ($n = 1$) or two isolates ($n = 1$) were acquired (Table 1). In total, 132 isolates were available for typing studies, among which 18 different PCR-ribotypes were observed. PCR-ribotype A15 was found in five (18 %) of 28 faecal samples. PCR-ribotypes A2, A3, A6 and A8 were each isolated from two or more patients, with each patient admitted to a different hospital department. Of the 23 patients with a first episode of CDAD, two (7 %) patients' faecal samples (10 and 21a, Table 1) contained two different PCR-ribotypes in the same sample (Fig. 1). From four patients (numbers 7, 12, 17 and 21, Table 1) more than one faecal sample from the same diarrhoeal period was obtained. Two faecal samples from patient 17 showed isolates that were PCR-ribotype A14, whereas the isolate from the third sample (17c, Table 1) was identified as PCR-ribotype A15. Patients 7, 12 and 21 had identical PCR-ribotypes in consecutive faecal samples. All 132 isolates were *tcdA*-positive and *tcdB*-positive and only patient 4, with PCR-ribotype A4, carried an isolate resistant to clindamycin (Table 1).

Table 1. Characteristics of 23 patients with a first episode of CDAD and PCR-ribotyping results of the isolates from them

Patient/sample no.	Gender	Age	Department	CDAD	Relapse	No. of isolates studied	PCR-ribotype*	*tcdA* profile†	*tcdB* profile‡	*erm*(B) profile§
1	M	46	Nephrology	Mild	–	5	A1	+	+	–
2	F	76	Outpatient	Mild	–	5	A2	+	+	–
3	M	71	Internal medicine	Severe	–	5	A3	+	+	–
4	M	50	Internal medicine	Severe	+	5	A4	+	+	+
5	M	61	Outpatient	Mild	–	5	A5	+	+	–
6	F	48	Haematology	Mild	–	5	A6	+	+	–
7a	F	69	Outpatient	Severe	–	2	A7	+	+	–
7b						5	A7	+	+	–
8	F	65	Outpatient	Mild	–	3	A8	+	+	–
9	M	62	Gastroenterology	Mild	–	5	A2	+	+	–
10	M	75	Infectious disease	Severe	–	2	A9	+	+	–
						3	A10	+	+	–
11	F	60	Gastroenterology	Mild	+	5	A11	+	+	–
12a	F	48	Nephrology	Severe	–	5	A8	+	+	–
12b						5	A8	+	+	–
13	F	13	Paediatrics	Severe	–	5	A12	+	+	–
14	F	51	Outpatient	Mild	–	5	A6	+	+	–
15	F	40	Gastroenterology	Mild	–	5	A3	+	+	–
16	M	77	Urology	Mild	+	5	A13	+	+	–
17a	M	13	Paediatric surgery	Mild	+	5	A14	+	+	–
17b						4	A14	+	+	–
17c						5	A15	+	+	–
18	M	56	Outpatient	Severe	–	4	A15	+	+	–
19	M	75	Intensive care	Mild	–	5	A15	+	+	–
20	M	77	Outpatient	Mild	–	5	A15	+	+	–
21a	F	73	Outpatient	Mild	+	4	A16	+	+	–
						1	A15	+	+	–
21b						5	A16	+	+	–
22	F	79	Neurology	Mild	+	5	A17	+	+	–
23	M	75	Intensive care	Mild	+	4	A18	+	+	–

*Designation of PCR-ribotypes using codes assigned in this study.

†PCR for detection of the *tcdA* gene.

‡PCR for detection of the *tcdB* gene.

§PCR for detection of the *erm*(B) gene.

Table 2. Characteristics of 23 patients with recurrent CDAD and PCR-ribotyping results of the isolates from them

Hospital patient no.	Age	Gender	Department	No. of episodes	PCR-ribotype*	*tcdA* profile†	*tcdB* profile‡	*erm*(B) profile§
I-1	41	M	Internal medicine	2	B1	+	+	–
					B2	+	+	–
I-2	1	M	Paediatrics	2	B3	–	–	–
					B4	+	+	+
I-3	61	M	Outpatient	2	B5	+	+	–
I-4	76	F	Intensive care	2	B3	–	–	–
I-5	37	M	Internal medicine	2	B6	+	+	–
I-6	78	M	Intensive care	2	B3	–	–	+
I-7	66	M	Surgery	2	B1	+	+	–
I-8	60	M	Internal medicine	2	B7	+	+	–
I-9	20	F	Intensive care	2	B1	+	+	–
I-10	81	M	Intensive care	3	B10	+	+	–
					B11	+	+	–
I-11	62	F	Surgery	2	B12	+	+	–
I-12	5	M	Paediatrics	2	B3	–	–	+
					B13	–	–	–
I-13	75	F	Outpatient	4	B7	+	+	–
					B8	–	–	–
					B9	+	+	–
I-14	52	M	Intensive care	2	B14	–	–	–
II-1	68	M	Internal medicine/oncology	2	B7	+	+	–
II-2	70	M	Vascular surgery	2	B17	+	+	–
II-3	83	M	Cardiac surgery	2	B15	+	+	–
II-4	66	M	Intensive care surgery	2	B16	+	+	–
II-5	78	F	Vascular surgery	2	B7	+	+	–
III-1	78	M	Outpatient	4	B18	+	+	–
III-2	60	F	Gastroenterology	2	B10	+	+	–
III-3	49	F	Outpatient	2	B7	+	+	–
III-4	13	M	Paediatric surgery	3	B19	+	+	–
					B20	+	+	–

*Designation of PCR-ribotypes using codes assigned in this study. Each different PCR-ribotype was isolated from a different faecal sample.

†PCR for detection of the *tcdA* gene.

‡PCR for detection of the *tcdB* gene.

§PCR for detection of the *erm*(B) gene.

Table 3. Primer sequences of oligonucleotides used for PCR-ribotyping and conventional PCR in this study

Primer	Nucleotide sequence (5′–3′)	Gene	Fragment length (bp)	Reference
PRBs*	GTGCGGCTGGATCACCTCCT	ITS†	Variable	Bidet *et al.* (1999)
PRBas	CCCTGCACCCTTAATAACTTGACC			
NKV011	TTTTGATCCTATAGAATCTAACTTAGTAAC	*tcdA*	2535	Kato *et al.* (1999)
NK9	CCACCAGCTGCAGCCATA			
NK104	GTGTAGCAATGAAAGTCCAAGTTTACGC	*tcdB*	204	Kato *et al.* (1998)
NK105	CACTTAGCTCTTTGATTGCTGCACCT			
282BacS	GAAAARGTACTCAACCAAATA	*erm*(B)	639	Sutcliffe *et al.* (1996)
283BacAS	AGTAACGGTACTTAAATTGTTTAC			

*PRB, PCR-ribotyping by Bidet.

†ITS, internal spacer region.

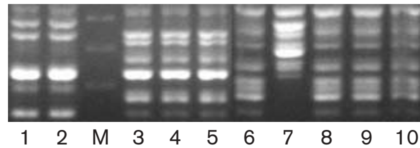

Fig. 1. PCR-ribotyping results of two patients with multiple PCR-ribotypes in one faecal sample. Lanes 1–2, two isolates from patient 10 belonging to PCR-ribotype A9; lane M, markers for 500, 400 and 300 bp; lanes 3–5, three isolates from patient 10 belonging to PCR-ribotype A10; lanes 6 and 8–10, four isolates from patient 21a belonging to PCR-ribotype A16; lane 7, one isolate from patient 21a belonging to PCR-ribotype A15.

Typing of *C. difficile* isolates from 52 faecal samples of 23 patients with recurrent CDAD

Of 23 patients with recurrent episodes of CDAD, 19 patients had two episodes, two patients had three episodes and two patients had four episodes (Table 4). The mean age of patients with recurrent CDAD was 55·7, varying between 1 and 83 years of age. Thirty percent of these were female and 70 % were male (Table 4); this did not differ significantly from the 23 patients with a first episode of CDAD. Of the 19 patients with one recurrence, two were outpatients, as were both of the two patients with three recurrences, whereas no outpatients were present among the two patients with two recurrences. Symptom-free intervals varied between a mean of 6·2 weeks (range 3–14) for the first interval, to a mean of 13·5 weeks (range 2–25) for the second, and a mean of 10 weeks (range 3–17) for the third symptom-free interval. The mean second symptom-free interval was longer than the mean first symptom-free interval, when comparing the groups of patients with different number of recurrences (Table 4).

In total, 20 different PCR-ribotypes were observed (Table 2). The most common PCR-ribotypes in faecal samples of the 23 patients with recurrent CDAD were PCR-ribotypes B7 and B3, present in faecal samples of five (22 %) and four (17 %) patients, respectively (Table 2). In hospital I, 14 types were found among 31 *C. difficile* isolates; the most common PCR-ribotype was type B3 in four of the 14 (29 %) patients. However, the *tcdA*- and *tcdB*-negative PCR-ribotype B3 was found to be *erm*(B)-negative in patients I-2 and I-4, whereas patients I-6 and I-12 had *erm*(B)-positive isolates (Table 2). In hospital II, four different PCR-ribotypes were found. Of the five patients from this hospital, two had PCR-ribotype B7 in their faecal samples. *C. difficile* isolates from four patients in hospital III belonged to five different PCR-ribotypes.

Of 23 patients with recurrent CDAD, six (26 %) showed a different PCR-ribotype isolate in a recurrent episode (Table 2). This is not significantly different from two of 23 patients with multiple *C. difficile* types detected in a first episode of CDAD (chi-square test, $P \leqslant 0{\cdot}2$). Of these six patients, patient I-13 harboured three different genotypes. Patient III-4 carried two toxinogenic strains, and is the same patient as patient 7 from Table 1. A total of three PCR-ribotypes were found in this patient: A14 (the same type as B20), A15 and B19; all three strains were toxinogenic (Tables 1 and 2). Patients I-2 and I-13 carried both toxinogenic and non-toxinogenic isolates, whereas patients I-4, I-6, I-12 and I-14 had only non-toxinogenic isolates in their faecal samples (Table 2). Clindamycin resistance was found in three of the 23 patients with recurrences (patients I-2, I-6 and I-12, Table 2).

DISCUSSION

Using PCR-ribotyping, toxinogenicity and clindamycin resistance, multiple types of *C. difficile* were found in two of 23 (8·7 %) patients with a first episode of CDAD. Additionally, six of 23 (26 %) patients with recurrent *C. difficile* infection had different types in their consecutive episodes. No significant difference was found in the presence of multiple types within one faecal sample and the occurrence of multiple types in recurrent CDAD.

The finding that multiple types of *C. difficile* were present in faecal samples in 8·7 % of 23 patients with a diarrhoeal

Table 4. Characteristics of 23 patients with recurrent episodes of CDAD

	First recurrence	Second recurrence	Third recurrence
Total number of patients	19	2	2
Mean age	54.4	47	76.5
Gender M/F	13/6	2/0	1/1
Inpatients	17	2	0
Outpatients	2	0	2
Mean first symptom-free interval (weeks)	6.2	7.5	8.5
Mean second symptom-free interval (weeks)	–	13.5	13.5
Mean third symptom-free interval (weeks)	–	–	10
No. of patients with different PCR-ribotypes (no. of types)	3 (2)	2 (2)	1 (3)
No. of patients with isolates with different toxin profiles	2	0	1
No. of patients with *erm*(B)-positive strains	2	0	0

episode is not in agreement with the findings of three previous reports using REA and randomly amplified polymorphic DNA (RAPD) fingerprinting (Wilcox *et al.*, 1998; O'Neill *et al.*, 1991; Devlin *et al.*, 1987). This difference could be due to the fact that three different plates and a combination of untreated and ethanol-treated faecal samples were applied in our study for selection of colonies. In contrast, colonies were only selected from a primary selective culture plate without an ethanol pre-treatment of faecal samples in other studies (O'Neill *et al.*, 1991; Devlin *et al.*, 1987). Two further studies were in agreement with our observations. Sharp & Poxton (1985) reported that two of three selected faecal samples contained different strains of *C. difficile* by immunochemical fingerprinting of *C. difficile* surface antigens. This observation was probably associated with the high number of different colonies ($n = 8$) investigated from each faecal sample. Borriello & Honour (1983) showed the concomitance of a cytotoxigenic and a non-cytotoxigenic *C. difficile* strain in seven faecal specimens of patients with clinical symptoms of CDAD which were at first diagnosed as non-cytotoxinogenic by cytotoxicity assays. In our study, all isolates of the 23 patients with a first episode of CDAD were *tcdA*- and *tcdB*-positive, but only faecal samples with a positive enzyme-linked fluorescence assay for TcdA were included.

Of 23 patients with recurrences, six (26 %) had culture-positive episodes with *tcdA*- and *tcdB*-negative isolates (Table 2). Moreover, one patient (I-12) had two episodes with *tcdA*- and *tcdB*-negative *C. difficile*. One explanation could be that these strains are capable of producing another toxin. In addition to the two large clostridial toxins (TcdA and TcdB), some strains of *C. difficile* also produce an actin-specific ADP-ribosyltransferase, called binary toxin CDT. The frequency of binary toxin genes among *C. difficile* strains that do not produce large clostridial toxins was reported to be 15·5 % in one study (Geric *et al.*, 2003). The binary toxin has cytotoxic effects on Vero cells, and may act as an additional virulence factor together with the large clostridial cytotoxins. Another possibility is that the six patients were simultaneously infected with a toxin-producing strain that was not cultured. We favour this explanation, since TcdA was detected in the recurrent episodes by an enzyme immunoassay. It also confirms the findings of the study by Borriello & Honour (1983) that concomitance of cytotoxigenic and non-cytotoxigenic *C. difficile* strains occurs.

Recurrences of CDAD occur in 15–20 % of cases after discontinuation of treatment (Wilcox & Spencer, 1992). In our study encompassing an observation period of 2 years, a recurrence rate of 30 % was found among the 23 patients with a first episode of CDAD. Once recurrent episodes develop, 45–60 % continue to have repeated episodes (McFarland *et al.*, 2002). Using PCR-ribotyping, our reinfection rate could be estimated as 26 %; this is lower than has been shown in other studies, in which the percentage of reinfection was found to be between 33 and 75 % (Tang-Feldman *et al.*, 2003; Barbut *et al.*, 2000; O'Neill *et al.*, 1991).

Relapses can be due to the persistence of spores not completely eradicated by therapy. Discrimination between reinfections and relapses is difficult if a particular strain is widespread in the environment and reinfects patients. Wilcox & Spencer (1992) showed that 56 % of recurrences were reinfections, using the RAPD method to fingerprint strains from 27 patients from six different hospitals. They also found, however, that an endemic clone of *C. difficile* accounted for 53 % of all isolates, and they hypothesized that the frequency of reinfections was probably underestimated because of the reacquisition of the same strain from the hospital environment. We included patients with recurrent CDAD from three different hospitals and found no endemic clone. In addition, patients can also contaminate their own environment by shedding the strain of the first episode, and subsequently become reinfected with the same strain. Finally, from the results of our current study we conclude that differentiation between reinfection and relapse on microbiological grounds is also difficult to determine, since patients may have been infected simultaneously with multiple types. Whether this will be recognized depends on the culture methods and number of colonies selected from different culture media for further typing studies.

No significant differences in age, gender, or in- and out-patient numbers were observed among the 16 patients with a single episode of CDAD in comparison with the 27 patients with recurrent CDAD. This is in contrast with previous studies (Young *et al.*, 1986; Fekety *et al.*, 1997; McFarland *et al.*, 1999; Do *et al.*, 1998). Young *et al.* (1986) investigated 35 patients and found a significant difference in age and a history of recent abdominal surgery. Fekety *et al.* (1997) and McFarland *et al.* (1999) performed a retrospective analysis of risk factors for CDAD, and a prospective analysis during a 2 month study. Female gender, an onset of the initial episode in spring, the number of previous episodes and antibiotic treatment for another infection shortly after a CDAD episode were significantly associated with recurrent CDAD (Fekety *et al.*, 1997). Two other risk factors predictive of recurrent CDAD were increasing age and a decreased quality-of-life score at inclusion (McFarland *et al.*, 1999). Chronic renal insufficiency, a high white-blood-cell count and community-acquired diarrhoea of the first episode have also been significantly associated with recurrent CDAD (Do *et al.*, 1998). This discrepancy with our findings may be due to the fact that we compared patients with a first episode diagnosed at one hospital in 2002 with patients suffering from recurrent episodes who were diagnosed in a period of 15 years at three different hospitals.

In summary, the simultaneous presence of multiple *C. difficile* PCR-ribotypes in faecal samples from patients with a first episode and recurrent CDAD did not differ significantly. This observation limits the application of typing methods for studying the exogenous or endogenous source of recurrences.

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