Genotyping of Outbreak-Related and Sporadic Isolates of *Clostridium difficile* Belonging to Serogroup C

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Received 18 March 1996/Returned for modification 30 April 1996/Accepted 23 September 1996

Serogroup C of *Clostridium difficile* is the serogroup most frequently related to outbreaks. Fifty-six toxigenic serogroup C isolates of *C. difficile* were genotyped by ribotyping PCR (ribo-PCR), random amplified polymorphic DNA (RAPD) assay, and pulsed-field gel electrophoresis (PFGE). Thirty-five of the 56 isolates were recovered from four unrelated outbreaks (Belgium, 1987, 1992, and 1995; France, 1992 to 1993), 7 derived from a spatiotemporal cluster in Cotonou, Benin (1992), and 14 were sporadic isolates. The serogroup C reference strain, also isolated during an outbreak (Belgium, 1983), was genotyped too. Ribo-PCR, the RAPD assay, and PFGE generated 2, 5, and 11 major genotypes, respectively. Combination of the three methods finally yielded 13 general types, although ribo-PCR did not play any role in enhancing resolution. Three general types were recovered from all the isolates from the five outbreaks and the cluster, with two types being predominant. The 14 sporadic serogroup C isolates were divided into 11 overall genotypes. These results indicate that genotyping methods, and more particularly the combination of the RAPD assay and PFGE, can resolve genetic diversity within toxigenic, serogroup C *C. difficile* strains. Also, this study suggests that outbreak-related serogroup C strains are limited to a few genetically stable and apparently very widely (internationally and intercontinentally) distributed genotypes.

Clostridium difficile plays a major role in intestinal diseases ranging from antibiotic-associated diarrhea to pseudomembranous colitis (4, 18). In the adult, nearly all cases of *C. difficile* infection occur after antibiotic therapy and, to a lesser degree, chemotherapy, with spontaneous cases being exceptional (10, 16, 40). In nonhospitalized asymptomatic adults, carriage is uncommon (0 to 3%) (26, 39). On the other hand, prevalence in hospitals is particularly high (10 to 15%) (30). Environmental contamination with spores and transmission by patient-to-patient contact and by medical staff, together with the high number of susceptible patients, maintains endemicity and has already caused numerous hospital outbreaks (5, 8, 14, 24). Currently, *C. difficile* is the major cause of nosocomial diarrhea (24).

Research to understand the epidemiology of this anaerobic bacterium has already resulted in various phenotyping and, more recently, genotyping methods (32). Serotyping has proved to be simple and rapid (10, 11, 36). It distinguishes 10 major serogroups (A, B, C, D, F, G, H, I, K, and X), some of which are readily related to pseudomembranous colitis and antibiotic-associated diarrhea while others are related to silent carriage in neonates (12, 14, 36). Serogroup C is by far the serogroup most frequently implicated in outbreaks.

In an attempt to study the genetic diversity and refine the epidemiology of this particular serogroup, we submitted 56 toxigenic serogroup C strains from our *C. difficile* collection to three different genotyping approaches: ribotyping PCR (ribo-PCR) (6, 13), the random amplified polymorphic DNA (RAPD) assay (3, 19), and pulsed-field gel electrophoresis (PFGE) (17, 22). Thirty-five isolates were collected during four unrelated outbreaks in different cities and countries, seven isolates represented a spatiotemporal cluster, and 14 isolates

were unrelated strains. The reference strain of each serogroup was also typed.

MATERIALS AND METHODS

Strains. The *C. difficile* reference strains of the 10 serogroups (A, B, C, D, F, G, H, I, K, and X) and a total of 56 toxigenic *C. difficile* isolates belonging to serogroup C were genotyped. Of these 56 isolates, 35 isolates were from four different outbreaks. An outbreak was defined as a significant increase in the temporal and local frequency of antibiotic-associated diarrhea accompanied by the isolation of toxigenic, serogroup C strains. Fifteen isolates were isolated during an outbreak in a cardiovascular surgery unit in our university hospital, St. Luc Hospital in Brussels, Belgium, between February and April 1995 and included six environmental isolates (bed rail, telephone, sink, floor, and knob). Another two contemporary toxigenic serogroup C isolates were added from symptomatic patients in the neighboring unit on the same floor and in a unit one floor below. Four isolates were from an outbreak in a hospital in Malmédy, Belgium, that occurred between May and September 1992. Four isolates were recovered from an outbreak in a home for elderly people in Dinant, Belgium, between December 1986 and December 1987, and 10 were recovered from patients in an infectious disease unit in a hospital in Annecy, France, between September 1992 and July 1993. Finally, seven isolates were recovered from the university hospital in Cotonou, Benin (in Africa), including one environmental isolate, but in this setting a clear relationship to an outbreak could not be established because of the high endemicity of *C. difficile*. We included these seven isolates in the outbreak group, as they nevertheless represented a temporal and geographical cluster of symptomatic patients on antibiotherapy: all were isolated within a 2-month period in a few wards of the same university hospital. It should be noted that the serogroup C reference strain (ATCC 43596), which was added to the study, had been isolated at the time of an outbreak in Namur, Belgium, in 1983 (10).

Fourteen toxigenic, serogroup C strains were selected as epidemiologically unrelated: six came from St. Luc Hospital at different times, i.e., from 10 years to 1 year before our outbreak, three came from the same French hospital in Annecy but 2 to 3 years later than their 1992 to 1993 outbreak, and five came from other hospitals or private laboratories in Belgium.

Identification. The strains were identified by their morphological aspect and gas chromatography. An in vitro cytotoxin production assay was performed on confluent monolayers of HeLa cells with and without *Clostridium sordellii* antitoxin after 5 days of growth in anaerobic liquid medium at 37°C (10). Strains were conserved in thioglycolate medium at room temperature.

Serotyping. Serotyping was carried out by slide agglutination with different antisera according to the method described by Delmée et al. (10).

Disc susceptibility testing. Disc susceptibility testing results were used as phenotypic markers as previously described for *C. difficile* (9). Standard discs of erythromycin (15 µg), rifampin (15 µg), chloramphenicol (30 µg), tetracycline (30 µg), and clindamycin (2 µg) were used. Zone sizes were measured after 48 h

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of incubation and for each antimicrobial agent, strains were classified as susceptible, intermediate, or resistant.

Extraction of DNA for ribo-PCR and the RAPD assay. DNA was extracted as previously described with slight modifications (3). Briefly, isolates were grown in 10 ml of prerduced peptone-yeast extract-glucose broth for 18 h at 37°C in an anaerobic atmosphere. After centrifugation at 4°C, cells were washed in TES buffer (10 mM Tris [pH 8.0], 50 mM EDTA, 150 mM NaCl), repelleted at 4°C, and resuspended in 5 mg of lysozyme per ml in TES buffer. Cells were lysed for 1 h at 37°C and for another 30 min after addition of sodium dodecyl sulfate (SDS; final concentration, 1%). Finally, the lysate was digested for 2 h at 37°C by adding pronase E to a final concentration of 1 mg/ml. DNA was purified first with 2 volumes of phenol-chloroform-isoamyl alcohol (25:24:1) and then with 1 volume of chloroform-isoamyl alcohol (24:1). After precipitation by cold ethanol and resuspension of the dried pellet in TE2 (10 mM TRIS, 2 mM EDTA), DNA quantity and quality were assessed by spectrophotometry (GeneQuant II; Pharmacia Biotech, Brussels, Belgium).

Ribo-PCR. For the ribo-PCR assay, primers complementary to conserved regions in the 16S and 23S genes of the rRNA operon were used to amplify the 16S to 23S intergenic spacer region (20, 21). PCR was performed in 50- μ l volumes containing 10 mM Tris-HCl (pH 9), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 200 μ M each deoxynucleoside triphosphate, 25 pmol of each primer, 100 ng of template DNA, and 1 U of DNA-free, *Taq* DNA polymerase (Applicene-oncor, Strasbourg, France). After an initial denaturation for 5 min at 94°C, 30 reaction cycles (30 s at 94°C, 30 s at 55°C, 30 s at 72°C) were performed, and this was followed by a final extension step of 7 min at 72°C.

RAPD assay. A single arbitrary primer, AP4 (5'-TCACGCTGCA-3'), was used for the RAPD assay (3). PCR assay conditions were identical to those for ribo-PCR, except for a higher MgCl₂ concentration (2.5 mM). Amplification consisted of an initial 5-min denaturation at 94°C, 35 reaction cycles (1 min at 94°C, 1 min at 36°C, and 2 min at 72°C), and a final 7-min extension step at 72°C.

Ribo-PCR and the RAPD reactions were carried out in a Gene Amp, model 2400 (Perkin-Elmer, Zaventem, Belgium). Amplification products were separated on a 2% agarose gel and stained with ethidium bromide. Interpretation by visual inspection was performed by two independent persons. Every two-band or greater difference with respect to a previous profile was considered a major type. For the 56 study strains, major ribotypes were assigned a lowercase letter and major RAPD types were assigned a Greek letter. Subtypes, i.e., those with a one-band difference, were indicated by a superscript number following the major type indicator (e.g., RAPD assay subtypes α^1 and α^2).

PFGE. For the PFGE analysis, isolates were incubated for 5 h in 10 ml of peptone-yeast extract-glucose broth at 37°C in an anaerobic atmosphere. Organisms were pelleted at 4°C and washed in TES buffer. Cells were resuspended in TES buffer and adjusted to 3×10^9 bacteria per ml. This suspension was mixed with the same volume of 1.2% low-melting-point agarose and poured into molds. It was allowed to solidify at 4°C. Plugs were pulled out and incubated in 1 mg of pronase E per ml in TE-SDS (10 mM Tris, 0.5 M EDTA, 1% SDS) overnight at 37°C. After eliminating pronase by several washes in TE2, DNA was digested with 15 U of *Sma*I for 4 h at 30°C. The resulting macrorestriction fragments were separated in a 1% agarose gel by PFGE (CHEF DRII; Bio-Rad, Nazareth, Belgium) at 6 V/cm, with a linear ramping from 20 to 50 s for 20 h. *Sma*I-digested *Staphylococcus aureus* (NCTC 8325) genomic DNA was used as the molecular size marker. After electrophoresis, DNA was stained with ethidium bromide. Again, visual inspection was done by two independent persons. Each major restriction profile was defined by scoring every four or more bands against a previous profile. The 56 study strains were designated by a Roman numeral. The major pulsotypes were eventually subtyped (here indicated by lowercase letters following the Roman numeral) when three or fewer band differences were noted (35).

RESULTS

Ribo-PCR. The 10 reference strains displayed 10 different major ribotypes (Fig. 1A), consisting of six to nine bands varying from approximately 500 to 1,000 bp. Two major ribotypes (a and b) were generated by the 56 serogroup C toxigenic strains, both of which consisted of eight bands in the same size range as those of the reference strains (Fig. 1b). As one ribotype was the same as the ribotype of the reference strain of serogroup C, it started the series and was thus designated ribotype a. It was found in 55 of the 56 strains (Table 1). Ribotype b, corresponding to none of the reference ribotypes, derived from one sporadic strain from a Belgian hospital.

RAPD assay. The assay generated seven major types for the reference strains (Fig. 2A). Serogroups G, H, I, and K presented only very faint band differences and were classified as the same major RAPD type. Reference serogroups A, B, C, D, F, and X each generated a unique type. RAPD typing of the 56

FIG. 1. (A) Ribo-PCR analysis of the reference strains of the 10 serogroups (indicated above the lanes) of *C. difficile*. (B) Representative examples of each major type, a and b (as indicated above the lanes), derived by ribo-PCR analysis of the study strains. A 1-kb DNA ladder (Gibco BRL, Merelbeke, Belgium) was used as molecular size marker (lanes m in panels A and B).

study strains (excluding the reference strain) resulted in five major RAPD types (α to ϵ). Two of these five types were subtyped, as they produced small, but reproducible differences: type α^1 showed the same pattern as type α^2 , but one band was significantly more intense (very strong versus faint); type β^2 differed from type β^1 by one extra band of medium intensity (Fig. 2B). Again, one of the RAPD types was identical to the RAPD type of the reference strain serogroup C and started the series as type α^1 . No other study strain type was identical to one of the other reference strain types. All the strains from the four outbreaks and from the Cotonou cluster belonged to type α or β (Table 1). Type α contained 12 strains from two outbreaks, the African cluster, and one sporadic strain. Type β comprised 30 strains from three outbreaks and 11 sporadic strains, of which 8 were locally (i.e., from the same ward or hospital) but not temporally related to two of those outbreaks. The remaining three types represented three sporadic strains.

PFGE. PFGE generated nine major pulsotypes for nine reference strains (Fig. 3A). Each digest yielded 10 to 13 fragments ranging from 10 to 700 kb. Serogroup G was repeatedly non-typeable because of DNA degradation. The study isolates (excluding the reference strain) were divided into 11 major pulsotypes, and one of these pulsotypes was subdivided into five subtypes (Fig. 3B). Isolates which generated the same profile as the reference strain of serogroup C were designated Ia. All the isolates of the four outbreaks and the Cotonou cluster fell into major type I (subtypes a, b, c, and d) or major type II. All of the 17 isolates from the outbreak in St. Luc Hospital belonged to type Ia. Isolates from the outbreak in Dinant and the African cluster generated both type Ib and type Ic, which differed from each other by one band. Type Ia differed from type Ib and type Ia differed from type Ic by two and three bands, respectively. Type Id and type Ia, both of which were recovered from the French outbreak, differed by two bands.

TABLE 1. Epidemiology and genotyping results of toxigenic serogroup C *C. difficile* strains

Strain	Nature ^a	Place of isolation ^b	Mo/yr of isolation	Origin	Ribo-PCR type	RAPD type	PFGE type	General type	Antibiotic susceptibility ^c
1	O	Brussels (1)	02/95	Patient	a	β^1	Ia	2	R
2	O	Brussels (1)	02/95	Patient	a	β^1	Ia	2	R
3	O	Brussels (1)	02/95	Patient	a	β^1	Ia	2	R
4	O	Brussels (1)	02/95	Patient	a	β^1	Ia	2	R
5	O	Brussels (1)	02/95	Patient	a	β^1	Ia	2	R
6	O	Brussels (1)	02/95	Patient	a	β^1	Ia	2	R
7	O	Brussels (1)	03/95	Patient	a	β^1	Ia	2	R
8	O	Brussels (1)	03/95	Patient	a	β^1	Ia	2	R
9	O	Brussels (1)	03/95	Patient	a	β^1	Ia	2	R
10	O	Brussels (1)	04/95	Patient	a	β^1	Ia	2	R
11	O	Brussels (1)	04/95	Patient	a	β^1	Ia	2	R
12	O	Brussels (1)	03/95	Environment	a	β^1	Ia	2	R
13	O	Brussels (1)	03/95	Environment	a	β^1	Ia	2	R
14	O	Brussels (1)	03/95	Environment	a	β^1	Ia	2	R
15	O	Brussels (1)	03/95	Environment	a	β^1	Ia	2	R
16	O	Brussels (1)	03/95	Environment	a	β^1	Ia	2	R
17	O	Brussels (1)	03/95	Environment	a	β^1	Ia	2	R
18	O	Malmédy	05/92	Patient	a	β^1	II	3	I (t)
19	O	Malmédy	06/92	Patient	a	β^1	II	3	R
20	O	Malmédy	09/92	Patient	a	β^1	II	3	R
21	O	Malmédy	09/92	Patient	a	α^1	Ia	1	R
22	O	Dinant	12/86	Patient	a	α^1	Ib	1	R
23	O	Dinant	12/87	Patient	a	α^1	Ib	1	R
24	O	Dinant	12/87	Patient	a	α^1	Ic	1	R
25	O	Dinant	12/87	Patient	a	α^1	Ic	1	R
26	O	Annecy	09/92	Patient	a	β^1	Ia	2	R
27	O	Annecy	09/92	Patient	a	β^1	Ia	2	R
28	O	Annecy	10/92	Patient	a	β^1	Ia	2	R
29	O	Annecy	10/95	Patient	a	β^1	Ia	2	R
30	O	Annecy	01/93	Patient	a	β^1	Id	2	R
31	O	Annecy	02/93	Patient	a	β^1	Id	2	R
32	O	Annecy	03/93	Patient	a	β^1	Ia	2	R
33	O	Annecy	03/93	Patient	a	β^1	Id	2	R
34	O	Annecy	05/93	Patient	a	β^1	Ia	2	R
35	O	Annecy	06/93	Patient	a	β^1	Ia	2	R
36	C	Cotonou	01/92	Patient	a	α^1	Ic	1	R
37	C	Cotonou	01/92	Patient	a	α^1	Ic	1	R
38	C	Cotonou	02/92	Patient	a	α^1	Ic	1	R
39	C	Cotonou	02/92	Patient	a	α^1	Ic	1	R
40	C	Cotonou	02/92	Patient	a	α^1	Ic	1	R
41	C	Cotonou	03/92	Patient	a	α^1	Ib	1	R
42	C	Cotonou	02/92	Environment	a	α^1	Ic	1	R
43	S	Brussels (1)	03/85	Patient	a	γ	IV	5	R
44	S	Brussels (1)	09/89	Patient	a	β^1	Ia	2	R
45	S	Brussels (1)	03/90	Patient	a	β^1	III	4	R
46	S	Brussels (1)	05/91	Patient	a	β^1	Ia	2	R
47	S	Brussels (1)	02/92	Patient	a	β^1	Ie	2	R
48	S	Brussels (1)	03/94	Patient	a	β^1	V	6	I (c)
49	S	Brussels (2)	06/91	Patient	a	α^2	VII	8	R
50	S	Brussels (3)	02/95	Patient	b	ϵ	XI	13	S (c,r)
51	S	Annecy	11/94	Patient	a	β^2	Ia	2	R
52	S	Annecy	03/95	Patient	a	δ	Ia	7	S (c)
53	S	Annecy	03/95	Patient	a	β^1	VI	12	R
54	S	Mouscron	02/92	Patient	a	β^1	VIII	9	S (c,cc,e,r)
55	S	Mouscron	01/95	Patient	a	β^1	X	11	S (c,cc,e,r)
56	S	Auvelais	04/94	Patient	a	β^1	IX	10	S (e,r)
Reference C	O	Namur	05/83	Patient	a	α^1	Ia	1	R

^a O, outbreak; C, cluster; S, sporadic.^b Brussels (1), (2), and (3) represent three different hospitals in Brussels; Brussels (1) is St. Luc Hospital.^c R, resistant to chloramphenicol, clindamycin, erythromycin, rifampin, and tetracycline; I (t), intermediately susceptible to tetracycline; S (c,cc,e,r), susceptible to chloramphenicol, clindamycin, erythromycin, and rifampin, respectively.

Three of the outbreak strains of Malmédy generated type II, and one generated type Ia. Types II and Ia differed from each other by six bands. Five sporadic strains (three from St. Luc Hospital and two from Annecy) also generated major type I

(four of subtype Ia and one of subtype Ie). Type Ie, represented by one strain from St. Luc Hospital isolated in 1992, differed from Ia (found in the outbreak in St. Luc Hospital) by two bands. The remaining nine major types derived from nine

FIG. 2. (A) RAPD analysis of the reference strains of the 10 serogroups (indicated above the lanes) of *C. difficile*. (B) Representative examples of each major type and subtype, α^1 through ϵ (as indicated above the lanes), derived by RAPD analysis of the study strains. A 1-kb DNA ladder (Gibco BRL) was used as a molecular size marker (lanes m in panels A and B).

sporadic strains, and each differed by more than six bands from type I (including subtypes) and type II. Types VIII and IX, as well as types IX and X, had two-band differences (insertion and deletion possibility, respectively), but type VIII and type X had a four-band difference. We attributed a major type indicator to each strain, as there was no apparent epidemiological link between these three strains.

General typing. When the individual results of the three typing methods were combined, 13 general types were generated (subtypes were not considered), which were designated by single digits. Type 1 (α , a, and I), which was identical to the combined RAPD assay, ribo-PCR, and PFGE results of the reference strain of serogroup C, accounted for 12 isolates involved in three outbreaks: all the strains of the Dinant outbreak, all the clinical and environmental strains of the Cotonou cluster, and one of the four strains of the Malmédy outbreak. Type 2 (β , a, and I) comprised 31 isolates: all the clinical and environmental strains from the outbreak in St. Luc Hospital, all the strains from the outbreak in Annecy, 3 sporadic strains from St. Luc Hospital (1989, 1991, and 1992), and 1 sporadic strain from Annecy (1994). Type 3 (β , a, and II) comprised three out of the four strains of the outbreak in Malmédy. The remaining 10 general types (types 4 to 13) corresponded to 10 sporadic strains. Seven of them (types 4 and 6 to 11) each had a different and unique pulsotype (III and V to X) but the same RAPD types and ribotypes (i.e., α or β and a, respectively) when compared with the general outbreak types (types 1 to 3). Type 12 had only a different and unique RAPD type (δ) but had the same ribotype and pulsotype (a and I, respectively). Type 5 had a different and unique RAPD type and pulsotype (γ and IV, respectively) but had the same ribotype (a). Lastly,

type 13 was of a unique type according to all three methods (ϵ , b, and XI).

Antibiotic susceptibility testing. All the serogroup C isolates were resistant to tetracycline, except for one strain from the outbreak in Dinant, which had an intermediate level of susceptibility to tetracycline (Table 1). The outbreak and cluster strains were all resistant to the other four antibiotics (chloramphenicol, clindamycin, erythromycin, and rifampin). Eight of the 14 sporadic strains were also multiresistant, and four of these multiresistant strains were of general type 2. The remaining six sporadic strains had five variable susceptibility patterns: intermediately susceptible to chloramphenicol, susceptible to chloramphenicol, susceptible to chloramphenicol and rifampin, susceptible to erythromycin and rifampin, and susceptible to chloramphenicol, clindamycin, erythromycin, and rifampin.

FIG. 3. (A) PFGE of *Sma*I restriction digests of the reference strains of the 10 serogroups (indicated above the lanes) of *C. difficile*. (B) Representative examples of each major type and subtype, Ia through XI (as indicated above the lanes), generated by PFGE of *Sma*I restriction digests of the study strains. A *Sma*I chromosomal digest of *Staphylococcus aureus* NCTC 8325 was used as a molecular size marker for PFGE analysis (lanes m in panels A and B).

DISCUSSION

Currently, genotyping methods are extensively used for their greater power of discrimination and their broader applicability compared with those characteristics of phenotyping methods. In our study, genotyping methods were able to enhance discrimination when they were applied to a group of *C. difficile* isolates previously phenotyped as toxigenic serogroup C. Also, our genotyping results seem to indicate that a clonal relatedness exists among toxigenic, serogroup C strains recovered from temporally and locally unrelated outbreaks but not among sporadically isolated strains.

General type 1, which was the type of the reference strain, was found in two Belgian outbreaks (Dinant and Malmédy) and in the Cotonou cluster (Table 1). These isolates were recovered over a 9-year period. General type 2 was found in two outbreaks, in France and Belgium. Moreover, this same type was found among four sporadic strains, all of which were isolated long before or after the outbreaks but in the same two hospitals. On the whole, this general type was recovered over a 6-year period. Intra- and interhospital spread is not a new finding but has been limited until now to a national area (6, 15, 23). Considering the high level of discrimination obtained with the remaining 10 sporadic strains (10 unique general types), the international and even intercontinental recovery, as well as the persistence over several years, of general types 1 and 2, seems to reflect a high and widely spread endemicity of those types and not a failure of the typing methods. This trend has already been described for certain clones of *S. aureus*, which spread more easily and remain genotypically constant (37).

These endemic strains, on the other hand, seem to be more readily related to outbreaks. The narrow relationship between endemicity and epidemicity is in a way not surprising. Numerous authors have already reported that nosocomial acquisition of *C. difficile* is facilitated by the persistence of its spores on environmental surfaces, by spread through personnel or patient-to-patient contact, and by intensive antibiotic therapy (8, 14, 15, 16, 22, 30). All our environmental strains in two outbreaks were of the corresponding clinical types. Yet, an additional potential source of contamination is asymptomatic patients, who can carry *C. difficile* for several weeks and perpetuate the chain of transmission during subsequent admissions in the same or other hospitals and tertiary-care hospitals (8, 14, 16, 24, 28, 39, 40). Although it was impossible to verify transfers among the different institutions in our study, this could be a possible explanation of the spread of general types 1 and 2.

Each of the three different genotyping approaches used in this study was previously evaluated for *C. difficile*, but not every possible comparison among these typing methods has been described so far. Ribo-PCR proved to be the best method for typing the reference strains of the 10 serogroups, since it was capable of differentiating all 10 strains. The RAPD assay also typed all the strains but was less discriminating, as it could not distinguish between serogroups G, H, I, and K. Using the same primer, AP4, Barbut et al. (3) found similar results, the use of a different polymerase probably accounting for the small differences (29, 38). Our PFGE results were in agreement with those of Kato et al. (17): PFGE was very discriminating, generating nine major pulsotypes for nine serogroups, but it lacked 100% typeability, serogroup G being untypeable because of DNA degradation. Recently, other authors generated a PFGE profile for the reference strains of all 10 serogroups, yet no profile was obtained for three of their study isolates (33).

Discrimination among the 15 sporadic strains was achieved

well by PFGE and almost as well by the RAPD assay, whereas the combination of the two typing methods increased the level of resolution (Table 1). Other authors found the RAPD assay to be nearly as discriminative as PFGE, but the RAPD types were the combined results of two or three different RAPD assays (7, 23, 34, 37). A combination of two different PFGE assays in order to increase resolution has also been reported (33). When applying strictly our type and subtype definitions, we were sometimes confronted by attribution dilemmas. Two strains (no. 54 and 55) that were locally related but temporally unrelated differed by four bands, each thus representing a major type. A third strain (no. 56), locally and temporally unrelated to strains 54 and 55, differed from these strains by only two bands. We finally categorized these strains by three major type indicators, since no epidemic link was apparent and the antibiotic susceptibility patterns were divergent. Nevertheless, the relatively small number of band differences compared with the long interval between recovery (26 months and 9 months) and the variability of phenotype expression, as well as the endemic character of *C. difficile* (these strains are nationally related), could indicate a common ancestor rather than a lack of discrimination.

All serogroup C strains within a single outbreak or cluster of toxigenic serogroup C *C. difficile* gave the same RAPD type, ribotype, and pulsotype, except those from the outbreak in Malmédy. Three of those four strains presented the same unique general type 3, confirming the epidemic link, but one strain had different RAPD and PFGE types. Yet, general type 1 of this strain was identical to that of the outbreak strains in Dinant and those of the Cotonou cluster. Thus, this fourth strain apparently took no part in the contemporary outbreak as was supposed earlier but was still tied to an outbreak-related general type. Multiple endemic strains of *C. difficile* existing simultaneously in the same hospital have already been reported by other authors (22).

The combination of the RAPD assay and PFGE increased the level of resolution of strains in the outbreak and cluster groups (Table 1). Again, interpretation of the PFGE results posed a problem; we designated five subtypes within type I on the basis of differences of no more than three bands from the bands of index strain Ia. But it is worth noting that comparing subtypes Ib through Ie with each other could result in differences of as much as five bands (e.g., subtypes c and d). As these differences were never found in the same outbreak or cluster, subtype designation was maintained. They could reflect different genetic divergences from the same ancestor.

The difficulties we encountered when designating types and subtypes highlight the fact that guidelines, although very helpful, need to be flexible if they are to be applied to a particular epidemic context (population size or study period). Also, typing results should always be compared with classical descriptive data (1, 35).

The discrimination power of ribo-PCR has been found to be high by other authors or at least as discriminating as other PCR-based typing methods (6, 13, 23). These findings and the excellent discrimination among the reference strains of the 10 serogroups are in contrast to the poor level of discrimination by this method among our study strains. We found a unique ribotype for all but one of the study strains. This seems to indicate that within the group of toxigenic serogroup C strains, the distributions of the rRNA intergenic spacer region alleles are similar and very stable. Whether this relationship is extensible to the other serogroups, toxigenic or not, remains to be studied, but Cartwright et al. (6) detected 41 distinct ribotypes among 102 isolates of *C. difficile* strains. However, the frequencies of distribution of the PCR ribotypes were unequal, and

relatively few ribotypes accounted for the majority of toxigenic isolates. The clinical significance of their and our findings remains to be elucidated. A recent study of *Streptococcus pyogenes* using, among other tests, the more classical restriction fragment length polymorphism ribotyping to subtype prevalent M serotypes causing invasive disease showed that various degrees of genetic heterogeneity existed, depending on the serotype studied (31).

Serogroup C strains present a typical resistance pattern to erythromycin, rifampin, chloramphenicol, tetracycline, and clindamycin, which can be exploited as a phenotypic marker (9). We found a nearly total correlation between toxigenic serogroup C and tetracycline resistance. General types 1, 2, and 3, from the outbreaks and cluster, were all resistant to the five antibiotics, whereas 6 of the 10 remaining sporadic general types showed susceptibility to at least one antibiotic, representing five unique patterns. These results suggest a relationship between genotype, resistance pattern, and (epidemic) virulence. Highly resistant, serogroup C strains were also found by other authors to be predominant in AIDS patients (2).

In summary, our findings seem to indicate that, among toxigenic serogroup C *C. difficile* strains, some very widely spread (internationally and intercontinentally) and genetically stable strains exist. They are, moreover, particularly virulent, resistant, and capable of generating outbreaks when they encounter a cluster of susceptible hosts in a more or less favorable environment. The existence of such genotypes could greatly influence decision-making and policy choice in terms of screening, isolation, disinfection, and (prophylactic) treatment (12, 14, 39). Therefore, more extensive and prospective studies are needed to confirm our results. Recently, a comparable outbreak relationship of a restricted number of genotypes was hypothesized for *Legionella pneumophila* (25).

Genotyping methods are precious tools in epidemiologic studies of *C. difficile*. PFGE is the most discriminatory typing method, but it is time-consuming and presents the problem of DNA degradation, which seems particularly prevalent among clostridial isolates (17, 22). The RAPD assay is technically easier and more rapid, but it is often not sufficiently discriminatory. Increased resolution is obtained when it is combined with other RAPD assays, which limits its advantages of speed and low cost. Ribo-PCR also proves to be technically simple, and the results are easier to interpret than those obtained by the RAPD assay. Because ribo-PCR detects characterizable and stable genetic variations, it is a more robust method and provides more reproducible results than the RAPD assay. Nevertheless, its discrimination power variably increases, perhaps depending on the serogroup(s) studied. In our study, i.e., within toxigenic serogroup C, the combination of PFGE and the RAPD assay gave the highest level of discrimination and ribo-PCR did not improve resolution.

ACKNOWLEDGMENT

We thank A. Sédailan (Annecy) for providing strains.

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