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PCR Ribotyping and Arbitrarily Primed PCR for Typing Strains of *Clostridium difficile* from a Polish Maternity Hospital

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Detection of the source of *Clostridium difficile* strains is of importance for the control of the nosocomial spread of this microorganism. For this purpose, vaginal and rectal swabs from 183 mothers, duplicate fecal samples (taken on days 1 and 4 after birth) from 183 neonates, and 94 environmental samples were cultured for *C. difficile*. The microorganism was never detected in the meconium obtained on day 1 after birth. On the other hand, an incidence of 17% *C. difficile* positivity was noted in the fecal samples obtained on day 4 after birth. Forty-two percent of the 31 colonized neonates had been delivered with complications. The bacteria were never encountered in the rectal swabs of the mothers, and *C. difficile* was identified in only one vaginal swab. In contrast, 13% of the environmental samples were positive for *C. difficile*. No major difference was encountered between patient and environmental isolates with respect to toxigenicity (58 to 65% toxigenic isolates). All strains were subsequently typed by PCR amplification of the 16S-23S ribosomal intergenic spacer regions and by arbitrarily primed PCR (AP-PCR) with different primers and combinations thereof. All environmental isolates and 11 of 31 neonatal strains were of a single type. The vaginal strain was unique, and among the maternity ward- and neonate-related isolates, only two additional AP-PCR types were identified. When a collection of *C. difficile* strains from patients hospitalized in other institutions and suffering from antibiotic-associated diarrhea or pseudomembranous colitis was analyzed in a similar manner, it appeared that the strain from the maternity ward was unique. The other strain commonly encountered among the neonates was also identified frequently among the isolates from patients with antibiotic-associated diarrhea or pseudomembranous colitis, indicating its general occurrence. On the basis of both epidemiological studies and PCR-mediated genotyping, it was shown that the environment and not the birth canal is the major source of *C. difficile* acquisition by neonates in this maternity hospital setting. Furthermore, AP-PCR appears to be a fast and useful method for epidemiologically relevant typing of *C. difficile* isolates.

The involvement of *Clostridium difficile* strains in antibiotic-associated diarrhea (AAD), colitis, and pseudomembranous colitis (PMC) has been well established (3, 5). The disease process seems to be highly complex, and the putative role of the two major toxin proteins, toxin A and toxin B, has attracted a great deal of attention (6, 29, 36). On the other hand, many healthy infants have been shown to carry *C. difficile* in their intestines (23, 34). Silent carriage of *C. difficile* is common during the first year of life, with rates ranging between 20 and 60% (13, 17), demonstrating the feasibility of asymptomatic *C. difficile* colonization. The role of this colonization in the development of disease and the mechanisms of resistance of healthy infants to this toxin-producing microorganism still remain obscure. Transmission of *C. difficile* strains from innate environmental surfaces has been reported previously (19, 35). The microorganism has been found in a wide variety of domestic and wild animals and in environmental niches including sand, soil, mud, and hay. As a result of this nearly ubiquitous presence, its capacity to cause significant clinical morbidity and mortality among patients that are on antibiotic treatment will be of even increasing concern in the near future (32). Also, the production of toxin, which is the major bacterial virulence factor causing morbidity and sometimes even mortality in adults (21), strongly adds to the pathogenicity of *C. difficile.*

The comparison of *C. difficile* strains isolated from different environmental and clinical sources is a prerequisite for detailed epidemiological studies. Several molecular-epidemiological typing procedures that can be applied to many microbial species have been developed (25). Most of these procedures can be successfully applied to *C. difficile* strains as well and appear to be useful for the elucidation of interstrain relationships. Recently, arbitrarily primed PCR (AP-PCR) (26, 33, 37, 41) and PCR ribotyping (8, 14, 15) have been used successfully for the latter purpose as well.

The aim of the present study was to determine the incidence of *C. difficile* infection and to compare *C. difficile* strains isolated from different sources within a Polish maternity hospital. Epidemiological surveillance by *C. difficile*-specific cultivation was followed by PCR-mediated typing of the strains that were encountered upon routine analysis of fecal samples from neonates, fecal samples and vaginal swabs from their mothers, and swabs from the maternity ward's environment.

MATERIALS AND METHODS

Epidemiological investigations. Culture was performed to isolate *C. difficile* from environmental swabs, fecal samples from neonates, and fecal and vaginal samples from the mothers of the neonates. The study was performed in a 72-bed maternity ward in a Polish hospital (Bielanski Hospital, Warsaw, Poland). The babies and the mothers were cared for in separate rooms. The mothers came into

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TABLE 1. Characterization of *C. difficile* strains isolated in the maternity hospital

Source of strains	No. of samples	No. (%) of cultures[a]: Positive	CA positive	AP-PCR group 1
Neonates				
Meconium	183	0		
Stool	183	31 (17)	20 (65)	13 (42)
Vaginal samples	183	1 (0.5)		
Mothers' stool	183	0		
Hospital environment	94	12 (13)	7 (58)	10 (83)
Medical personnel				
Feces	96	0		
Hands or fingernails	96	0		
Total	1,018	44	27	23

[a] CA, *C. difficile* cytotoxicity assay on McCoy cells; only the numbers of strains testing positive are indicated. For a definition of AP-PCR group 1, see the overall types in Table 2.

contact with the babies only at times of breast-feeding. The babies were transported and cared for by different nurses. The study period was 10 months. Samples were obtained from 183 pregnant women prior to delivery. Two fecal samples were obtained from their newborn children (n = 183 children). The first (meconium) sample was collected on day 1 after birth, and the second was obtained on day 4. Ninety-four environmental samples were collected within the maternity ward. Samples were obtained from neonates' bedrails, baby sinks, baskets for reusable diapers, surfaces of baby changing tables, the baby scale, and walls behind radiators. Fecal samples and swabs of hands and fingernails (n = 96) were obtained from the attending medical staff and nurses.

Fifteen additional *C. difficile* strains from 13 patients suffering from AAD or PMC were collected from seven different institutions and hospitals in Warsaw. Consecutive isolates (spanning approximately 2 months of time) from two patients were included. Strains were derived from different wards in only one of the institutions (locations Ia and Ib; see Table 3).

Bacterial strains. Identification of *C. difficile* was based on tests described previously (16, 27, 28). Samples were collected with cotton swabs and transported in a specialized medium (Transport Medium Set I; BioMed, Krakow, Poland). Inoculation of swabs was on another selective medium enriched with 0.1% sodium taurocholate with cycloserine-cefoxitin-amphotericin B (TCCA; Bio-Merieux, Marcy l'Etoile, France). Anaerobic incubation (Gasbox System; Bio-Merieux) was performed for 48 h at 37°C. Fecal samples and rectal swabs were processed similarly on brucella blood agar with TCCA. Vaginal and environmental samples were cultured in liquid brain heart infusion (BHI) broth with TCCA. Further identification of suspected *C. difficile* isolates was based on colony morphology and bacterial shape, as determined microscopically. Yellow-green fluorescence under UV light, biochemical tests, and the characteristic *p*-cresol smell (horse odor) were assessed before final species confirmation. The toxigenicity of the isolates was tested by cytotoxicity and neutralization assays with McCoy cells (27, 28, 30, 37). In short, strains were grown in BHI broth for 48 h at 37°C. After centrifugation and filtration the toxigenicity of the culture supernatant was tested in duplicate on McCoy cells. Several dilutions (10^{-1} to 10^{-8}) were tested, and cytotoxicity was considered positive if at least 50% of the McCoy cells adopted a rounded shape and if this activity could be neutralized by antitoxin. As controls, supernatants of toxigenic strain VPI 10463 and nontoxigenic strain NIH BRIGGS 8050 were used.

DNA isolation. The protocol used for DNA isolation has been described in detail before (24). Briefly, *C. difficile* isolates were cultured for 24 to 48 h in BHI broth. One milliliter of the resulting culture was centrifuged at 3,000 × g for 10 min, and the resulting bacterial pellet was resuspended in 200 μl of distilled water. The suspension was boiled for 10 min, residual bacterial debris was removed by centrifugation at 6,000 × g for 10 min at 4°C, and the supernatant fraction was either stored at −20°C or further processed for DNA purification by using chaotropic salts and Celite affinity chromatography (4).

AP-PCR. AP-PCR was performed as described previously (38). Primers were the enterobacterial repetitive intergenic consensus sequences ERIC1 and ERIC2 (39). The nucleotide sequences of these primers are 5′-ATGTAAGCTCCTG GGGATTCAC-3′ and 5′-AAGTAAGTGACTGGGGTGAGCG-3′, respectively. Two additional primers, which were also used previously (9), were used to corroborate the findings obtained with the ERIC primers. The sequences of AP2 and AP3 were 5′-TCATGATGCA-3′ and 5′-TCACGATGCA-3′, respectively. PCR consisted of two consecutive cycling programs. Initially, four cycles of 3 min at 94°C, 5 min at 35°C, and 5 min at 72°C were performed. Subsequently, a similar 40-cycle program was applied (40 s at 94°C, 1 min at 35°C, 2 min at 72°C). Amplified DNA was analyzed by electrophoresis in 1.5% agarose gels run in 0.5 × TBE (0.089 M Tris borate, 0.089 M boric acid, 0.002 M EDTA) at 100 mA of constant current. Interpretation of the banding patterns was done by visual inspection of the Polaroid pictures. Differences of even single bands were scored, and every different DNA banding pattern was indexed with a Roman numeral. The combined results of the individual assays were expressed as a single digit.

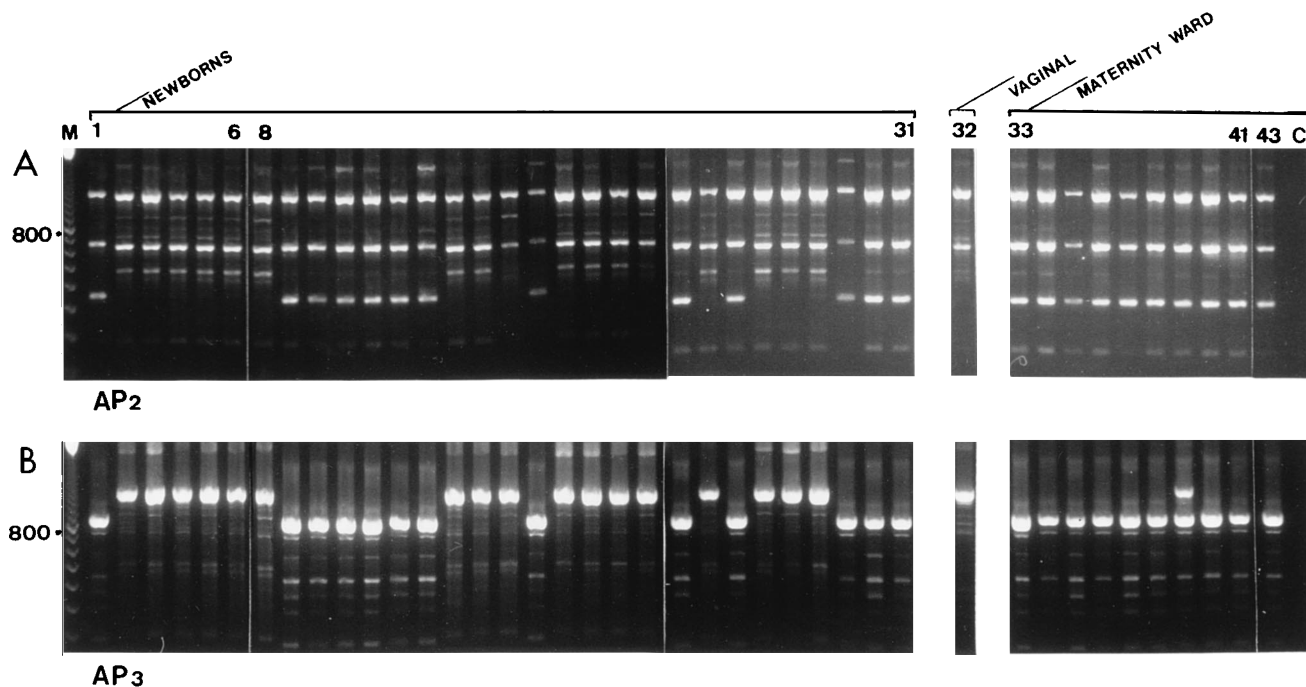

FIG. 1. AP-PCR of *C. difficile* strains from a maternity hospital. DNA was amplified with primer AP2 (A) or primer AP3 (B). Strains were derived from neonates or the maternity ward environment. A single vaginal strain is included. Numbering of the isolates is identical to that in Table 2. Lane M, molecular mass markers (100-bp ladder; Promega, Leiden, The Netherlands); the 800-nucleotide fragment is highlighted; lane C, result of amplification in the absence of extraneously added DNA.

TABLE 2. Survey of preliminary typing data of *C. difficile* from Polish hospitals[a]

Typing no.	Polish no.	Nature of strain	CA dilution	Type by AP-PCR with primer: ERIC1-ERIC2	AP2	AP3	Ribotyping spacer type	Overall type
1	3A	Newborns	10^{-4}	I	I	I	A	1
2	5A	Newborns	NT	II	II	II	B	2
3	13A	Newborns	10^{-4}	II	II	II	B	2
4	15A	Newborns	10^{-2}	II	II	II	B	2
5	35A	Newborns	10^{-5}	II	II	II	B	2
6	41A	Newborns	NT	II	II	II	B	2
7	53A	Newborns	10^{-5}	II	II	II	B	2
8	55A	Newborns	10^{-6}	II	II	II	B	2
9	61A	Newborns	10^{-6}	I	I	I	A	1
10	63A	Newborns	NT	I	I	I	A	1
11	75A	Newborns	10^{-4}	I	I	I	A	1
12	83A	Newborns	NT	I	I	I	A	1
13	85A	Newborns	NT	I	I	I	A	1
14	87A	Newborns	10^{-3}	I	I	I	A	1
15	97A	Newborns	10^{-4}	II	II	II	B	2
16	101A	Newborns	NT	II	II	II	B	2
17	139A	Newborns	NT	II	II	II	B	2
18	147A	Newborns	10^{-3}	I	I	I	A	1
19	153A	Newborns	10^{-5}	II	II	II	B	2
20	161A	Newborns	10^{-4}	II	II	II	B	2
21	223A	Newborns	NT	II	II	II	B	2
22	237A	Newborns	10^{-2}	II	II	II	B	2
23	261A	Newborns	NT	I	I	I	A	1
24	267A	Newborns	10^{-3}	II	II	II	B	2
25	283A	Newborns	NT	I	I	I	A	1
26	323A	Newborns	10^{-2}	II	II	II	B	2
27	329A	Newborns	NT	II	II	II	B	2
28	333A	Newborns	10^{-2}	II	II	II	B	2
29	335A	Newborns	10^{-2}	I	I	I	A	1
30	339A	Newborns	NT	I	I	I	A	1
31	343A	Newborns	10^{-2}	I	I	I	A	1
32	325	Vaginal	NT	III	III	III	C	3
33	8X	Bedrail	10^{-3}	I	I	I	A	1
34	9X	Basket	NT	I	I	I	A	1
35	12X	Sink	10^{-2}	I	I	I	A	1
36	18X	Surface	10^{-3}	I	I	I	A	1
37	19X	Basket	NT	I	I	I	A	1
38	21X	Wall	10^{-3}	I	I	I	A	1
39	37X	Bedrail	NT	I	I	IV	D	4
40	39X	Basket	10^{-3}	I	I	I	A	1
41	55X	Scale	NT	I	I	I	A	1
42	60X	Bedrail	NT	I	I	I	A	1
43	62X	Scale	10^{-2}	I	I	I	A	1

[a] Isolates 33 to 43 represent environmental strains; the other isolates were derived from single individuals. The environment in the maternity ward harbored a single strain of *C. difficile*. Only two strains were identified among newborns, and one of these strains represents the major type from the maternity ward (type 1; 13 of 31 strains). Primers ERIC1, ERIC2, AP2 and AP3 were described in Materials and Methods. CA, *C. difficile* cytotoxicity assay on McCoy cells; results are given as the highest dilution of the supernatant still inducing cytotoxic effects, as defined in Materials and Methods (NT, noncytotoxic). Underlining indicates a complicated delivery. Strains were equally divided across all possible groups when compared with the strains from noncomplicated deliveries. Strains from the maternity ward environment were derived from various inanimate surfaces (for additional information, see Materials and Methods).

PCR ribotyping. Analysis of the variations in lengths of the ribosomal intergenic spacer regions was performed as described before (8, 14). Use of primers SP1 (TTGTACACACACCGCCCGTCA, specific for the 16S rRNA gene) and SP2 (GGTACCTTAGATGTTTCAGTTC, specific for the 23S rRNA gene) in combination with a PCR program consisting of 40 cycles of alternating denaturation (1 min at 94°C), primer annealing (1 min at 55°C), and primer extension (1 min at 74°C) led to visualization of the amplified stretches of DNA after gel electrophoresis as described above. Every different banding pattern was assigned a capital letter. PCR ribotyping was performed only on the strains from the neonates, the mothers, and the maternity ward.

RESULTS

Epidemiological screening. Except for a single vaginal isolate (which was not found in the corresponding infant) *C. difficile* was not encountered in the rectal and vaginal swabs of the mothers. Likewise, no strains were encountered in the samples obtained from personnel. *C. difficile* was never detected in the meconium obtained from the neonates on day 1 after birth. On the other hand, in fecal samples obtained on day 4 after birth, the incidence of *C. difficile* colonization was 17%. All children appeared to be healthy at the time of sampling, without clinical signs of bacterial disease. Forty-two percent of 31 colonized neonates had been delivered with complications (delivery by Cesarean section, forceps delivery, malpresentation, etc.; see Table 2). Thirteen percent of the environmental samples derived from the baby rooms in the maternity ward were positive for *C. difficile*. Table 1 provides a

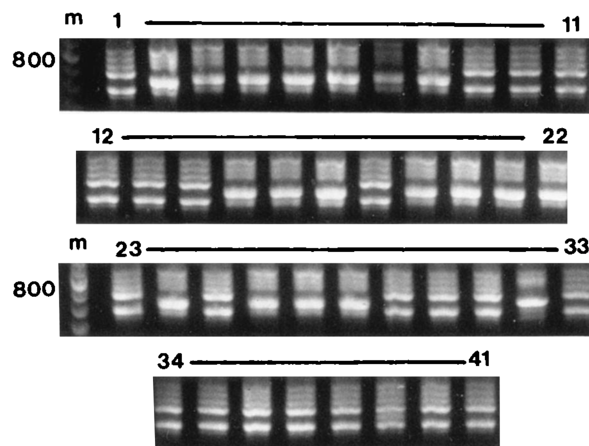

FIG. 2. PCR ribotyping of *C. difficile* strains from a maternity hospital. DNA was amplified with primers SP1 and SP2. Strains were derived from neonates or the maternity ward environment. A single vaginal strain is included (lane 32). Numbering of the isolates is identical to that in Table 2. Lanes m, molecular mass markers (100-bp ladder; Promega); the 800-nucleotide fragment is highlighted.

survey of all results. No major differences between patient and environmental isolates with respect to toxigenicity were encountered (Table 1). The respective proportions of toxigenic strains were similar (65 and 58%).

PCR analysis. From Fig. 1 it can be deduced that the results obtained with primer AP2 are fully corroborated by the data collected with primer AP3 except for the results for strain 39 (Polish strain 37X). This implies that the identities observed by two AP-PCR tests may be contradicted by the results of a third assay. Consequently, a single AP-PCR test may not be considered decisive in establishing clonality among *C. difficile* strains (see also Discussion section). The PCR fingerprints generated with the ERIC1-ERIC2 combination provide additional experimental confirmation (Table 2). When the results of the individual AP-PCR assays are combined into a single digit, only four different types were encountered among the isolates from the neonates and the maternity ward (including the single and unique vaginal strain). Interestingly, the ribotyping results are precisely concordant with the AP-PCR data (Table 2 and Fig. 2). Two overall *C. difficile* genotypes were found among strains isolated from newborns. Type 1 was encountered among 13 of 31 isolates, with the additional 18 strains belonging to genotype 2. Type 1 was also encountered among 10 of 11 of the environmental strains. This implies that a large portion of the strains from neonates were acquired in the ward shortly after delivery. The fact that the other newborns ($n = 18$) were colonized with strains with another, single genotype indicates the existence of an additional (probably also environmental) source in the separate department for newborns (see also below). Since this genotype was not identified among all of the environmental isolates, its source remains enigmatic. Isolate 39 (Polish isolate 37X), also from the maternity ward environment, displayed a different genotype (type 4); the AP3 AP-PCR and the ribotyping PCR concordantly showed aberrant banding patterns. On the other hand, the fact that the ERIC1-ERIC2 PCR and the AP2 AP-PCR showed that isolate 39 was identical to the other (genotype 1) strains might indicate that this single strain is identical to the majority of the other genotype 1 strains. The vaginal isolate appeared to be unique, which indicates, together with the epidemiological data, that the environment and not the birth canal is the major source of *C. difficile* transmission in this maternity hospital setting.

The results obtained with the group of *C. difficile* reference strains from the patients suspected of having AAD and PMC confirmed and strengthened the findings described above. Table 3 summarizes the typing data for this set of isolates and shows that the type 1 strains, found solely in the ward environment and a proportion of the colonized neonates, are unique for this hospital setting. This type 1 strain was not encountered in the reference panel. Type 2 strains, however, seemed to be quite common. This clone was present in three of seven institutions. In the hematology department in the Banacha Hospital, its presence persisted for at least 3 years (from 1991 to 1994). Persistence in an individual patient was demonstrated as well (isolates 25D and 25D3). Interestingly, this strain seemed to be capable of showing either a cytotoxic (isolate 25D) or a nontoxigenic (isolate 25D3) phenotype. This phenomenon is once more obvious from the data in Table 3, where it can be noted that both genotypes 1 and 2 can be present in either a toxigenic or a nontoxigenic form. Note that in the other duplicate set of strains from one individual the isolates were once more genetically identical (type 6 for strains 19D and 19D3). Among the strains from the 13 patients studied, 7 different *C. difficile* genotypes (types 2 and 5 through 10) were discernible.

DISCUSSION

Nosocomial disease caused by *C. difficile* is no rarity (2, 10, 18), and single-source outbreaks have been described before (12, 16). After a 6-month screening program performed in geriatric and medical wards it was concluded that about 80% of all *C. difficile* isolates belonged to a single, clonal type (7). Screening of a large group of newborns also revealed that single clones of *C. difficile* may be spread by person-to-person transmission through a common environmental source (23). It was demonstrated recently (31) that in a large tertiary referral hospital a high incidence of nosocomial *C. difficile* diarrhea does not necessarily imply the spread of a single bacterial clone. Nosocomial epidemics seem to be uncommon in this setting; in only a single instance limited clustering of a characteristic *C. difficile* genotype was observed. These data contradict those presented in two previous reports (7, 22) and also contradict our present observations. Although the study mentioned above did not include maternity or neonatology wards, it is still rather surprising that we encountered only two genotypes of *C. difficile* in a single department, one of which was unique to this particular setting. Although little is known about the spread of *C. difficile*, it is clear from our study and previous studies (11, 16, 18) that long-term contamination of the hospital environment can lead to a relatively high percentage of colonization in immunologically and microbiologically naive newborn infants.

Molecular typing of microorganisms is rapidly replacing more established techniques like biotyping and serotyping as the major experimental tool in the hands of hospital epidemiologists and microbiologists. The applicability and clinical relevance of the genetic approaches have also been documented for *C. difficile*. Restriction enzyme analysis, pulsed-field gel electrophoresis (PFGE) of DNA macrorestriction fragments, and ribotyping were shown to provide concordant data (22). Another genotypic approach, AP-PCR analysis (37, 40, 42), has been brought forward more recently. It was suggested and subsequently demonstrated that AP-PCR provides a rapid and sensitive screen for the determination of clonal relationships among *C. difficile* strains (1, 26). Comparisons between AP-PCR and immunoblotting (20) and between AP-PCR versus PFGE and ribotyping (9) underscored the value of AP-PCR: the resolution and epidemiological accuracy are strikingly

TABLE 3. Typing of *C. difficile* strains from patients in different hospitals and suffering from AAD or suspected of having PMC[a]

Strain identity	Location	Patient no.	Isolation date (day-mo-yr)	CA dilution	Type by AP-PCR with primer:			Overall type
					ERIC1-ERIC2	AP2	AP3	
3A					I	I	I	1
5A					II	II	II	2
325					III	III	III	3
37X					I	I	IV	4
17D	Ia	1	06-08-91	10^{-4}	II	IV	II	5
19D	Ib	2	31-07-91	NT	IV	ND	V	(6)[b]
19D3	Ib	2	29-09-91	NT	IV	V	V	6
21D	Ib	3	31-07-91	10^{-1}	II	II	II	2
24D	Ib	4	18-10-91	10^{-3}	II	II	II	2
29D	Ib	5	16-12-94	10^{-1}	II	II	II	2
210D	Ib	6	09-08-92	10^{-4}	II	II	II	2
25D	II	7	08-11-91	10^{-2}	II	II	II	2
25D3	II	7	20-12-91	NT	II	II	II	2
2S	III	8	30-10-90	NT	I	VI	VI	7
19S	III	9	20-12-90	NT	I	VI	VI	7
8D	IV	10	19-12-90	10^{-5}	V	IV	VII	8
11D	V	11	08-03-91	10^{-5}	II	VII	VIII	9
12D	VI	12	10-04-91	ND	VI	VIII	IX	10
13D	VII	13	22-04-91	10^{-4}	II	II	II	2

[a] The banding pattern codes are directly comparable to those mentioned in Table 2; this is also valid for the overall genocode. Since the data in Table 2 show that additional PCR ribotyping does not enhance the resolution, no spacer amplification was performed for the strains described here. The first samples displayed (3A, 5A, 325, and 37X) are also mentioned in Table 2; experiments, however, were performed in duplicate in order to determine the reproducibility of the assay and to enable in-gel comparison of the different banding patterns. The cytotoxicity is indicated by the supernatant dilution still displaying 50% cell shape changes in the McCoy cells. The location describes the following Warsaw hospitals and wards: I, Banacha; II, Mokotowski; III, Bielany; IV, Lindleya; V, Institute of Hematology, Warsaw; VI, Nieklanska; VII, Stepinska; a, pneumology ward; b, hematology ward; c, ophthalmology ward; d, surgery ward (when no lowercase letter is given, the origin of the strains is not precisely known). Other abbreviations: CA, cytotoxicity assay; NT, noncytotoxic; primers ERIC1, ERIC2, AP2, and AP3 were described in Materials and Methods; ND, not done.

[b] Overall type is in parentheses because of an incomplete data set (no results for AP2 PCR).

good. When 20 strains, shown by PFGE to be genetically distinct, were assayed by the three AP-PCR tests described in this report, 17 overall types could be identified by AP-PCR. Strains showing severe DNA degradation upon PFGE, a characteristic problem in PFGE typing of *C. difficile*, could be successfully typed by AP-PCR (data not shown; collaboration with M. Samore, Deaconess Hospital, Boston, Mass.). The studies mentioned above, however, are generally mere technical evaluations, and only a limited number of studies in which the molecular methods are used to gain insight into the nosocomial spread, persistence, and acquisition of *C. difficile* strains have been presented to date.

PCR ribotyping has recently been proposed as an effective means of studying *C. difficile* epidemiologically (8). The resolution is excellent and correlates well with data obtained by restriction enzyme analysis. We have shown here that the results of PCR ribotyping are also concordant with those of AP-PCR: both procedures classify the strains from maternity wards and neonates in an identical fashion. Since PCR ribotyping is considered more reproducible than AP-PCR, it may be preferable over the latter, technically more complex technology (15). This matter is subject to further investigation, as is the relationship between PFGE and AP-PCR. Interestingly, it appears that the AP-PCR banding patterns obtained by a given primer can vary among isolates, whereas the same isolates display identical fingerprints if other primer species are used (see, for instance, isolates 17D and 11D in Table 3). This suggests some degree of DNA mosaicism among *C. difficile* strains and necessitates the use of multiple AP-PCR assays in epidemiological surveys.

The fact that genotype 2 is frequently encountered in various Polish hospitals raises several questions. It might be that this *C. difficile* clone spreads more easily, has a better survival rate on inanimate objects, or displays enhanced colonization and/or infection efficiency. The strain also shows phenotypic variability. Further analysis of the biology of this clone and research on its national and international spread might be interesting options.

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