Application of chromosomal restriction endonuclease digest analysis for use as typing method for *Clostridium difficile*

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SUMMARY The usefulness of restriction endonuclease analysis of chromosomal DNA as a typing method for *Clostridium difficile* was tested. Over four months all faecal samples were routinely cultured for *C. difficile*. DNA of all isolated strains was isolated and tested with the restriction endonuclease Hind III. The patterns obtained after electrophoresis in agarose gels seemed to be strain specific. Antibiotic susceptibility profiles agreed with the results of the restriction endonuclease analysis, though they were much less discriminating. Analysis of the results indicated that restriction endonuclease analysis is a suitable typing method for *C. difficile*, which may be very valuable in epidemiological studies where a highly discriminating typing method is needed.

Clostridium difficile is a pathogen associated with diarrhoea and (pseudomembranous) colitis, which occurs nearly always after antimicrobial treatment. *C. difficile*, however, can also be cultured from a low percentage of healthy subjects¹ and from even higher percentages of asymptomatic inpatients.² *C. difficile* is also a common inhabitant of the gut in young children, apparently without causing disease.³

Some studies have shown that *C. difficile* is easily transmissible, evidenced by clustering of cases in wards in hospitals or in institutions.^{4,5} Likewise, the colonisation of neonates by *C. difficile* is now thought to originate from the environment and not from the vagina of the mother.⁶ The study of the epidemiology of *C. difficile* is, however, seriously hampered by the lack of sensitive and highly discriminating typing methods. Though different methods such as antibiogram typing,⁷ serotyping,⁸ typing by electrophoresis patterns of soluble cellular proteins with different modifications⁹⁻¹¹ or phage and bacteriocin typing¹² have been occasionally used with success, these methods are not always suitable for epidemiological studies, because they lack sensitivity or discriminating power, or because the required

reagents are not commercially available. Restriction endonuclease analysis of chromosomal DNA of bacteria has been used successfully in epidemiological studies of *Leptospira*,¹³ *Campylobacter*,¹⁴ and *Legionella*.¹⁵ We therefore decided to investigate the suitability of this method for epidemiological studies of *C. difficile*.

Material and methods

ISOLATION OF *C. difficile* FROM FAECES

Over four months all faecal samples sent to our laboratory for culture on enteropathogenic bacteria were plated on a selective medium for *C. difficile*. The selective medium used was brain heart infusion agar (Difco), supplemented with 5% (v/v) horse blood, 0.1% (w/v) sodium tauroglycocholate, cefoxitin (10 mg/l) and cycloserin (250 mg/l).^{16,17} The identity of all suspected isolates was confirmed by standard identification methods.¹⁸

CYTOTOXIN ASSAY

All faecal samples and all isolated strains were tested for the presence of cytotoxin or the ability to produce cytotoxin. Vero cells (African green monkey kidney cells, Flow Laboratories) were used in this assay, which was performed by standard methods.¹⁹

SUSCEPTIBILITY TESTING

All isolates were tested for their susceptibility to nine different antibiotics by the broth microdilution method. The antibiotics tested were: chloramphenicol, rifampicin, penicillin G (Gist Brocades, Delft, The Netherlands), cefotaxitin (Merck, Sharp and Dohme), clindamycin (Upjohn), vancomycin (Eli Lilly), metronidazole (Rhône-Poulenc), ticarcillin (Beecham) and piperacillin (Lederle). All drugs were used as commercially available materials. Dilutions of antibiotics were made in supplemented brain heart infusion broth.¹⁸ The trays were stored until use at -70°C , and before inoculation they were prereduced for four hours in an anaerobic chamber. After inoculation with diluted overnight cultures grown in brain heart infusion broth, yielding an inoculum of about 10^5 colony forming units (cfu) per well, the trays were incubated anaerobically for 40 hours.

The agar dilution method of susceptibility testing²⁰ was used for confirmation of the results for three antibiotics: chloramphenicol, clindamycin, and penicillin G.

FERMENTATION OF SORBITOL

Fermentation of sorbitol was tested in peptone-yeast extract medium¹⁸ with 1% (w/v) sorbitol. Tubes with 3 ml of the sorbitol medium were inoculated with 100 μl of turbid cultures (grown for 24 hours in brain heart infusion broth) of the strains to be tested, and incubated in an anaerobic chamber at 37°C for 48 hours, after which the pH was measured using a pH meter with a combination electrode (Russell, Helmstadt-Bargen, West Germany).

PREPARATION OF CHROMOSOMAL DNA OF *C. difficile* STRAINS

Essentially the same method as described by van Ketel *et al*¹⁵ was used. Bacterial cells were harvested from brain heart infusion broth cultures (100 ml), grown anaerobically for 72 hours by centrifugation and resuspended in 2.5 ml TES buffer (150 mM sodium chloride; 10 mM Tris; 10 mM sodium edetic acid; pH 8.0). After incubation for 60 minutes at 37°C with lysozyme (1 mg/ml) cells were lysed by the addition of sodium dodecylsulfate (SDS) in a final concentration of 1% (w/v), followed by an incubation period of 10 minutes at 60°C . The lysate was then incubated with ribonuclease A (Boehringer, Mannheim) in a final concentration of 100 $\mu\text{l}/\text{ml}$ for 60 minutes at 37°C , followed by digestion with proteinase K in a final concentration of 500 $\mu\text{g}/\text{ml}$ for 60 minutes at 60°C . After addition of sodium chloride to a final concentration of 1M the DNA was extracted with phenol-chloroform-isoamylalcohol (25:24:1) and precipitated with ethanol. The precipitated DNA was dissolved in saline-citrate buffer (0.015 M sodium

chloride; 0.0015 M trisodium citrate; pH 7.0) to yield a concentration of about 500 $\mu\text{g}/\text{ml}$.

RESTRICTION ENZYME DIGESTION OF DNA AND GEL ELECTROPHORESIS

About 8 μg DNA was digested with 8 U of the restriction enzyme Hind III in buffer (50 mM sodium chloride; 10 mM Tris; 10 mM magnesium chloride; 1 mM dithiothreitol; pH 7.5) at 37°C for two hours. Digests were then electrophoresed for 22 hours at 30 V in a 0.7% agarose gel (electrophoresis buffer: 40 mM Tris; 20 mM sodium acetate; 2 mM sodium edetic acid; 6 $\mu\text{g}/\text{ml}$ ethidium bromide; pH 7.7). The gel (20 $\times$ 20 cm) was then photographed with a Polaroid camera under ultraviolet illumination.

Results

During the study period 203 faecal samples were screened for the presence of *C. difficile* or its toxin. Thirty three specimens from 28 different patients were culture positive; only five of these samples were positive in the cytotoxin assay. As expected, samples from children aged less than one year were most often culture positive—16 of 29 (55%). A second group of patients in whom *C. difficile* was commonly found were those receiving oral antibiotics for selective gut decontamination: seven of 19 patients (37%) had a positive stool culture at least once during the study period. In the remaining group of patients only seven of 105 samples (7%) were culture positive. In view of these data it is not surprising that clustering of patients with positive cultures occurred only in the paediatric wards, where also most patients undergoing selective decontamination of the gut were nursed. No other clustering was observed during surveillance.

Table 1 shows data about origin (ward and patient) and characteristics of the strains (toxin production, antibiogram, and restriction endonuclease pattern).

ANTIBIOGRAM TYPING

The minimum inhibitory concentration values of the isolates were uniform. Table 2 shows the typical susceptibility profiles. Differences in susceptibility of one dilution step were disregarded. All isolates were tested at least twice, and reproducibility was excellent, except for chloramphenicol and clindamycin, where occasionally irregular growth in isolated wells was observed. Therefore we repeated the susceptibility testing for chloramphenicol, clindamycin, and penicillin G by an agar dilution method: reproducibility was good and the aberrant profiles were confirmed, though systematically lower minimum inhibitory concentration values were obtained for penicillin G and systematically higher minimum inhibitory concen-

Table 1 Origin and characteristics of *C difficile* strains

Strain No	Case No	Ward	Toxin production	Antibiotic profile	Restriction type
4*	1	6 M	+	I	A
6*	1	6 M	+	I	A
7*	1	6 M	+	I	A
10*	2	?	+	III	C
12*	3	?	+	II	D
15	4	2 O	+	I	
16	5	Outpatient	—	I	
17	6	Outpatient	+	I	
18	7	4 O	+	I	
19	8	10 M	+	I	
20	9	6 M	+	II	D
21	10	10 M	+	I	B
22	11	Outpatient	+	I	
23	12	Outpatient	+	III	C
24	13	10 M	+	I	B
25	14	8 W	+	I	B
26	15	10 W	+	I	
27	10	10 M	+	III	C
28	16	9 W	—	I	
29	10	10 M	+	I	B
30	8	10 M	+	I	
31	17	10 M	+	I	
32	18	Outpatient	+	I	
33	19	4 O	+	I	
34	17	10 M	+	I	B
35	20	9 W	+	I	B
36	21	8 O	+	I	
37	22	4 O	+	I	
38	23	8 M	+	I	
39	24	Outpatient	+	I	
40	25	10 M	+	I	B
41	26	4 O	+	IV	E
42	17	10 M	+	I	B
43	17	9 W	+	I	B
44	28	Outpatient	+	I	B
45	29	10 M	+	I	
46	26	4 O	—	I	
47	10	10 M	+	III	C

*Strains isolated prior to the study period.

tration values for chlor-amphenicol. It is obvious from these results that the discriminating power of antibiotyping for this set of isolates is low: only seven of 38 isolates (18%) showed an aberrant profile.

FERMENTATION OF SORBITOL

Sorbitol fermentation tests using different broth bases and different pH indicators yielded results that were

uninterpretable because of lack of clear cut positive or negative results. Finally, we measured the pH after incubation. Fig 1 shows that only quantitative differences in sorbitol fermentation exist between *C difficile* strains.

RESTRICTION ENDONUCLEASE ANALYSIS

In a preliminary study three restriction endonucleases

Table 2 Susceptibility profiles of 38 *C difficile* strains

Antibiotic tested	Profile No*					
	I	II	III	IV	Reference 21	Reference 22
Chloramphenicol	≤ 0.5 (4)	≥ 16 (≥ 128)	≤ 0.5 (4)	≤ 0.5 (4)	2-4	≤ 1
Rifampicin	≤ 0.06	≤ 0.06	≤ 0.06	≤ 0.06	≤ 0.06	≤ 1
Clindamycin	0.25 (0-12)	≥ 32 (≥ 128)	0.25 (0-12)	≥ 32 (≥ 128)	4-8	≤ 1
Vancomycin	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	2-4	≤ 1
Metronidazole	0.35	0.25	0.25	0.25	0.25-0.5	≤ 1
Cefoxitin	64	64	64	32	64-128	32-128
Ticarcillin	4	8	16	4	16-64	≤ 1
Piperacillin	4	4	8	2	4-16	≤ 1
Penicillin G	1	2	4	1	1-4	≤ 1
No of strains	31	2	4	1		

*Minimum inhibitory concentration values are in mg/l; values between parentheses are obtained by an agar dilution method.

Fig 1 pH values after 48 hours of incubation of strains in sorbitol fermentation broth: → designates pH of uninoculated broth.

were tested on the DNA of a few *C. difficile* strains: EcoR I, BamH I, and Hind III. Only Hind III digestion of DNA resulted in discrete patterns after electrophoresis, whereas digestion was incomplete with EcoR I and even more so with BamH I. Hind III digestion of the DNA of three isolates (cases 4, 6, and 7) from three stool samples of one patient during an episode of pseudomembranous colitis yielded identical patterns, designated as restriction pattern type A, thus showing the reproducibility of the method (fig 2). Another 35 isolates were then analysed with this method. Fig 3 shows an example of patterns of randomly chosen isolates. Inspection of all patterns after gel electrophoresis showed 22 different patterns. Ten isolates yielded completely similar patterns (restriction pattern type B, fig 4), and all these isolates were cultured from stool specimens of children. Nine of these children were patients in one of three paediatric wards. The tenth child was seen in the outpatient department; as a neonate this child had stayed two weeks in the maternity ward, four months earlier. A second group of four identical isolates (designated as restriction type C, fig 4) was found. These isolates showed a slightly lower susceptibility to penicillin G, ticarcillin, and piperacillin (antibiotic profile III).

Two of these isolates (cases 27 and 47) were isolated from the same patient (case 10) as were the two identical isolates (cases 21 and 29) of restriction pattern type B, indicating that this patient was colonised alternately, or perhaps simultaneously, with two different strains. A last group of only two identical isolates was designated as restriction pattern type D (fig 4). For comparison, a single isolate (restriction pattern type E; fig 4) is shown, as this shares the resistance against clindamycin but not against chloramphenicol with the two isolates of restriction pattern type D. All other strains showed unique electrophoresis patterns.

Discussion

Restriction endonuclease analysis of chromosomal DNA seems to be a suitable method for discriminating between individual strains of *C. difficile*. Our results strongly suggest that each individual strain shows a unique restriction endonuclease pattern. The fact that groups of isolates with an aberrant profile coincide with groups with identical restriction endonuclease patterns supports the validity of this

Fig 2 Agarose gel electrophoresis patterns of Hind III digests of chromosomal DNA. Lane 1: strain 4; lane 2: strain 6; lane 3: strain 7.

Fig 3 Agarose gel electrophoresis patterns of *Hind* III digests of chromosomal DNA of 17 *C difficile* strains (Lanes 1 to 17). Lane 18 represents *Hind* III digest of λ DNA. Lanes represent following isolates (lane number: isolate number): lane 1:46; lane 2:45; lane 3:40; lane 4:35; lane 5:33; lane 6:32; lane 7:31; lane 8:27; lane 9:26; lane 10:25; lane 11:17; lane 12:11; lane 13:7; lane 14:6; lane 15:15; lane 16:4; lane 17:10.

Fig 4 Agarose gel electrophoresis patterns of *Hind* III digests of chromosomal DNA of *C difficile* strains with restriction pattern type A (lane 1), B (lane 2), C (lane 3), D (lane 4) and E (lane 5).

method. With respect to discriminating power, restriction endonuclease analysis seems to be superior to other typing methods. Antibigram typing is not very discriminating, at least not in this study. Another drawback of antibiogram typing for sensitive anaerobic micro organisms is the existence of technical problems associated with this method, which was reflected by our own difficulties and by the discrepancies between the results of different studies using similar techniques.²¹ The same lack of discrimination is to be expected from serotyping, as in a study of 315 strains 99% could be typed with only six antisera, indicating that many different strains belong to the same serotype.⁸

We were not able to confirm the observation of Delmee, Homel, and Wauters⁸ that sorbitol fermentation is a useful discriminating characteristic of *C difficile* strains. The continuous distribution of pH values suggests that most strains are weak fermentors of sorbitol, with only quantitative strain differences, making sorbitol fermentation unsuitable for use as an epidemiological marker. The same results were found for the fermentation of xylose and trehalose (data not shown). The discrepancy between our results and those of Delmee *et al* cannot be explained by the different broth base that we used; 10 strains tested in thioglycolate broth as used by Delmee *et al*,⁸ pro-

duced similar results in peptone yeast extract medium (data not shown). Similar results using peptone yeast extract medium were obtained (data not shown).

Though this study was primarily intended to evaluate this typing method for *C. difficile*, some interesting results were obtained. The colonisation of children in three different wards with the same strain of *C. difficile* indicates that this strain must have been present in the environment in these wards in fairly high numbers for a prolonged period. This is indicated both by the fact that children were colonised with this particular strain throughout the four month survey period and by the fact that this strain was isolated in three neonates as early as the second day of life. That this strain was isolated in three different wards can be related to the transferral of children between these paediatric wards. Another possibility is spread of *C. difficile* by doctors visiting different paediatric wards. The high percentage of children colonised with this particular strain, while only occasionally other strains were isolated, suggests a difference in the ability of different strains to colonise the intestines as already suggested by Delmee *et al.*⁸ Another interesting phenomenon was the alternating isolation of two different strains of *C. difficile* from one patient (case 10). This patient received oral antibiotics for selective gut decontamination during treatment for leukaemia. The frequent isolation of *C. difficile* from this and similar patients suggests that patients receiving antibiotics for selective decontamination may be especially prone to colonisation by *C. difficile*. The importance of this, previously observed by Rogers *et al.*²³ is not yet clear and deserves further study.

These two examples show the suitability of restriction endonuclease analysis for epidemiological studies of *C. difficile*. This method, perhaps in combination with more simple but less discriminating typing methods, facilitates further studies in some aspects of the pathogenesis of *C. difficile* infections, such as mode of transmission or colonising ability, which depend on a highly discriminating typing method.

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