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 subjects and from even higher percentages of asymptomatic inpatients. *C. difficile* is also a common inhabitant of the gut in young children, apparently without causing disease.

Some studies have shown that *C. difficile* is easily transmissible, evidenced by clustering of cases in wards in hospitals or in institutions. Likewise, the colonisation of neonates by *C. difficile* is now thought to originate from the environment and not from the vagina of the mother. 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 anti-biogram typing, serotyping, typing by electrophoresis patterns of soluble cellular proteins with different modifications or phage and bacteriocin typing 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*, *Campylobacter*, and *Legionella*. 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). The identity of all suspected isolates was confirmed by standard identification methods.

**CYTOXIN 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.
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), cetotixin (Merck, Sharp and Dohme), clindamycin (Upjohn),
vancomycin (Eli Lilly), metronidazole (Rhône-Poulec), ticarcillin
(Beecham) and piperacillin (Lederle). All drugs were
used as commercially available materials. Dilutions of
antibiotics were made in supplemented brain heart
infusion broth. 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 testing 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 medium 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 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 lysoyme (1 mg/ml) cells were lysed by the addition
of sodium dodecyl sulfate (SDS) in a final concentra-
tion of 1% (w/v), followed by an incubation period
of 10 minutes at 60°C. The lysate was then incubated
with ribonuclease A (Bohringer, Mannheim) in a
final concentration of 100 µl/ml for 60 minutes at
37°C, followed by digestion with protease K in a
final concentration of 500 µg/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-isooamylalcohol (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 µg/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 µg/ml ethidium bromide; pH 7-7). The gel
(20 x 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
positive 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
positive 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 under-
going selective decontamination of the gut were
nursed. No other clustering was observed during sur-
veillance.

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 sus-
cceptibility 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 peni-
cillin G by an agar dilution method: reproducibility
was good and the aberrant profiles were confirmed,
though systematically lower minimum inhibitory con-
centration values were obtained for penicillin G and
systematically higher minimum inhibitory concen-
Chromosomal restriction endonuclease digest analysis for typing C difficile

Table 1  Origin and characteristics of C difficile strains

<table>
<thead>
<tr>
<th>Strain No</th>
<th>Case No</th>
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<th>Antibiotic profile</th>
<th>Restriction type</th>
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<td>10 M</td>
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</table>

*Strains isolated prior to the study period.

It is obvious 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.

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

<table>
<thead>
<tr>
<th>Antibiotic tested</th>
<th>Profile No*</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>I</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>≤ 0.5 (4)</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>≤ 0.06</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0.25 (0.12)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>≤ 0.5</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>0.25</td>
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<tr>
<td>Cefoxitin</td>
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</table>

*Minimum inhibitory concentration values are in mg/l; values between parentheses are obtained by an agar dilution method.
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.
Chromosomal restriction endonuclease digest analysis for typing C difficile

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. Antibiogram typing is not very discriminating, at least not in this study. Another drawback of antibiogram typing for sensitive anaerobic microorganisms 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. 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.

We were not able to confirm the observation of Delmee, Homel, and Wauters 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.
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 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.9 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.9 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.

References


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