Subtyping of *Clostridium difficile* PCR ribotype 001 by REP-PCR and PFGE

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The REP-PCR (repetitive sequence-based PCR using repetitive extragenic palindromic primers) typing method and a modified PFGE method were applied to isolates of *Clostridium difficile* PCR ribotype 001 with the aim of comparing their performance as methods of subtyping this organism. Of 200 isolates from 60 hospitals tested by REP-PCR, eight subtypes were identified and labelled as REP-PCR subtypes 001–008. The predominant subtype, REP-PCR subtype 003, accounted for 47 % of the total. Fifty-two of the 200 isolates were analysed by a modified PFGE method and seven subtypes were identified, labelled as PF-A–PF-G. There was excellent correlation between REP-PCR subtypes and PFGE subtypes with both methods displaying broadly similar discriminatory powers. However, REP-PCR subtyping proved to be a much easier, cheaper and more rapid method suitable for application for routine subtyping of *C. difficile* ribotype 001. Application of REP-PCR subtyping to UK isolates of *C. difficile* PCR ribotype 001 from 60 different centres revealed a wide distribution of REP-PCR subtype 003 throughout England and Wales, with a regional clustering of REP-PCR subtype 001 around Northwest England and North Wales. Analysis of isolates from a single hospital over a 4-year period revealed a change in predominant subtype over time.

**INTRODUCTION**

*Clostridium difficile* is recognized as the major cause of nosocomially acquired antibiotic-associated diarrhoea and pseudomembranous colitis (Bartlett, 1994; George *et al.*, 1978; Tabaqchali & Jumaa, 1995) and is a significant financial burden on modern healthcare resources (Wilcox *et al.*, 1996). Numerous *C. difficile* reservoirs exist within hospitals, including environmental surfaces, ward and surgical staff (Verity *et al.*, 2001) and colonized new admissions (Clabots *et al.*, 1992). This huge potential for infection of patients from diverse sources necessitates methods for typing *C. difficile* to understand the epidemiology of outbreaks and isolated cases, to identify any possible incidence of cross-infection and to set up surveillance programmes to monitor virulent strain emergence and hospital reservoirs.

Investigations performed at the Anaerobe Reference Laboratory (ARL) in Cardiff revealed that one strain, *C. difficile* PCR ribotype 001, accounted for 58 % of all hospital isolates tested (Brazier, 2001). This type appears to be endemic throughout most UK hospitals and is associated with both acute and prolonged outbreaks (Brazier & Borriello, 2000), causing serious outbreaks in the UK (Cartmill *et al.*, 1994) and overseas (al-Barrak *et al.*, 1999; Kato *et al.*, 2001; Rafferty *et al.*, 1998).

A subtyping study by Fawley *et al.* (2003) combined RAPD analysis, ribospacer PCR (RS-PCR) and PFGE to identify two subgroups within a small subset of PCR ribotype 001. Another study using pyrolysis mass spectrometry (PMS) identified nine major groups within ribotype 001 (Al-Saif *et al.*, 1998). However, they reported the inability of the PMS method to assign permanent types and the disadvantage of being unable to compare results between batches.

A recent study performed at the ARL using rep-PCR showed the presence of seven subtypes of ribotype 001 isolates using REP primers (Rahmati *et al.*, 2005).

The rep-PCR primers are complementary to repetitive sequences distributed throughout the genomes of many Gram-negative and some Gram-positive bacteria (Koeuth *et al.*, 1995). Using PCR, this method amplifies diverse regions of DNA flanked by the REP sequences, leading to amplicon patterns specific to particular subgroups within type 001.

PFGE is a method that has also been previously applied to the typing of *C. difficile* (Samore *et al.*, 1995; Sperner *et al.*, 1999). Considered by many as the ‘gold standard’ of typing methods, PFGE differentiates isolates based on the restriction patterns produced after whole genome digestion with an
polymerization mix, 2.5 mM MgCl2, 25 pmol REP1R-I primer, 25 pmol made up to 25

The PCR reaction was set up with minor modifications to a previously described method (Rahmati et al., 2005) to determine which method was most suitable for application to subtyping of C. difficile ribotype 001. Within these 200 isolates, a subset of 67 isolates from a single centre in Southeast England was subjected to REP-PCR typing to detect any changes in subtype over the years 1992–1996 inclusive.

METHODS

C. difficile strains. Two hundred isolates of C. difficile PCR ribotype 001 were randomly selected from isolates previously submitted to the ARL from 60 different hospitals between 1992 and 2004. This included 67 isolates from one hospital in Southeast England that formed a single-centre study to determine if a change in strains might occur over time. All isolates were previously identified as C. difficile by morphology, UV and latex testing (Brazier & Borriello, 2000). All had previously been typed as PCR ribotype 001 using an adaptation of a previously described method (Stubbs et al., 1999).

DNA extraction. Isolates were stored on Microbank beads (Prolab Diagnostics) at −80 °C. They were cultured on Fastidious Anaerobic Agar (FAA) supplemented with 5 % horse blood (Lab M) and incubated in an anaerobic atmosphere (10 % CO2, 10 % H2, 80 % N2, by vol.) at 37 °C for 18–24 h. Genomic DNA extraction was performed by making a suspension of a 1 µl loopful of cells in a 0.05 % (w/v) solution of Chelex-100 and boiling for 12 min. After centrifugation at 15 000 g.p.m. for 10 min, the supernatant was removed and used for the PCR reaction.

Primers and amplification conditions. REP primers REP1R-1, 5’-IIICGGCIGCATGCGG-3’, and REP2-1, 5’-IGCITTATCIGGCCTACG-3’, were used (Versalovic et al., 1991).

The PCR reaction was set up with minor modifications to a previously described method (Rahmati et al., 2005). The amplification mix was made up to 25 µl and contained 2.5 µl 10× PCR buffer, 800 µM dNTP polymerization mix, 2.5 mM MgCl2, 25 pmol REP1R-1 primer, 25 pmol REP2-1 primer (Sigma-Genosys), 2 units Taq polymerase (Amersham), 3 µl extracted template DNA (~60 ng DNA per reaction) and 16.1 µl water. A template-negative control was included in each set of reactions.

The PCR cycle was performed on a Peltier hot-bonnet thermal cycler (MJ Research) under the following conditions: 95 °C for 2 min, 94 °C for 3 s, then 35 cycles of 92 °C for 30 s, 40 °C for 1 min and 65 °C for 8 min, with a final extension step at 65 °C for 8 min. Analysis. PCR products were resolved on a 3 % Metaphor Agarose gel (Bio Whittaker) made with 1× TAE buffer (40 mM Tris, 20 mM glacial acetic acid, 1 mM EDTA; Bio-Rad) and ethidium bromide at 0.4 µg ml−1. PCR product (8 µl) was loaded and run with a 2 kb ladder at least every five lanes to allow correct normalization of gel image. Electrophoresis was performed at 150 V, 65 mA for 2.7 h. Banding patterns were visualized using Gel-Doc software (Bio-Rad), which generated a tagged image file format (TIFF) image used for analysis of the gels on Gel Compar II software version 4.1 (Applied Maths, Kortrijk, Belgium). To compare the relatedness of the banding patterns of each of the eight subgroups, we performed cluster analysis on the subtypes from one gel. The unweighted pair group method with arithmetic averages (UPGMA) on Gel Compar II software was used for the cluster analysis calculations.

PFGE and DNA extraction. Fifty-two strains were chosen at random from the 200 selected for REP-PCR. A modified PFGE method (Gal et al., 2005) was performed. Briefly, a number of crucial steps were identified that overcame the DNA degradation problems inherent with the original method. These included better lysis of the bacterial cells by increasing lysozyme concentrations and lysis incubation times, using mutanolysin in addition to lysozyme, and suspending the cells in lysis buffer rather than wash buffer prior to addition of agarose. Increasing the duration and concentration of proteinase K treatment was also found to be necessary to reduce smearing and DNA degradation. Formaldehyde fixation of cells on ice produced sharper bands and facilitated easier band interpretation.

RESULTS AND DISCUSSION

Our results support earlier research that subtypes exist within C. difficile ribotype 001 (Fawley et al., 2003; Al-Saif et al., 1998; Rahmati et al., 2005). Both subtyping methods gave 100 % typeability for all 200 isolates and the profiles generated were found to be highly reproducible between different operators and gels and over time regarding band number, relative position and intensity. Analysis of the banding patterns using REP-PCR revealed between 7 and 12 bands including 6 common bands at approximately 1940, 550, 510, 410, 340 and 320 bp (Fig. 1). The range of banding suitable for analysis was between 2000 and 246 bp and excluded one constant band above the 2 kb ladder. Isolates were assigned a new subtype if the banding patterns varied by the position of at least one reproducible band, or the presence or absence of one or more bands. Light and inconsistent bands were present in some gels but these were not included in the subtyping analysis.

Initial research showed the presence of seven REP-PCR subtypes from 50 isolates (Rahmati et al., 2005). After testing further isolates and refining the libraries and databases for the present study, eight reproducible subtypes were identified from 200 C. difficile isolates, numbered 001–008 (Fig. 1). Of the 200 isolates examined, 192 were UK isolates and 8 were foreign in origin, and these 200 represented 14.5 % (200/1384) of the total C. difficile PCR ribotype 001 isolates held in the ARL collection to date.

The most common profile was REP-PCR subtype 003, representing 47 % (95/200) of the isolates, while REP-PCR subtypes 001, 004 and 005 represented 9, 12 and 28.5 % of strains, respectively (Table 1). REP-PCR subtypes 002, 006, 007 and 008 formed four minor subtypes, accounting for 1, 0.5, 1 and 1 % of isolates, respectively.

Analysis of PFGE gels showed a range of banding patterns, containing between 9 and 11 bands, of which 5 bands were commonly shared. Isolates were assigned a new subtype if the banding patterns varied by the position of at least one band, or the presence or absence of one or more bands. PFGE analysis yielded a similar discriminatory power to REP-PCR.
with seven subtypes identified, labelled PF-A–PF-G (Table 1), and associations between the types recognized by REP-PCR and PFGE were strong. The most common group, PF-E, consisted of 22 isolates, 16 of which corresponded with REP-PCR subtype 003. PF-C, with 11 isolates, corresponded 100% with REP-PCR subtype 001 and PF-G corresponded completely with the four isolates of REP-PCR subtype 004. The two isolates of PF-D matched REP-PCR subtype 007 strains, while the two isolates in PF-B both corresponded with REP-PCR subtype 002. The unique PF-A corresponded with the single REP-PCR subtype 006 isolate.

The only major discrepancy between the two methods was within PFGE-subtype F. This group of 10 isolates was subdivided into four REP-subtypes: 003 (5/10), 004 (1/10), 005 (3/10) and 008 (1/10).

DNA degradation is a recognized problem of PFGE with some C. difficile isolates, including PCR ribotype 001 (Klaassen et al., 2002; Kristjansson et al., 1994; Hielm et al., 1998). The profiles generated with the modified PFGE method were easy to interpret and highly reproducible. However, the method remains complex and relatively expensive, costing approximately £8 sterling per isolate, per test. It is a lengthy protocol with a turnaround time of 5 days and requires specialist equipment to perform. In comparison, REP-PCR is much easier, quicker and cheaper to perform and has a much higher throughput capacity.

Either of the techniques described in this study could be applied to construct a subtyping scheme for C. difficile ribotype 001. Reproducibility and method standardization play an important role in the development of valid inter-laboratory typing schemes (Struelens et al., 1998). The issue of inter-gel variation using Gel Compar software has been addressed by Lefresne et al. (2003) and was found to be statistically insignificant when used within one laboratory. However, studies into the inter-laboratory reproducibility of rep-PCR methods and PFGE typing methods have shown that a lack of standardization can lead to poor reproducibility (Weigel et al., 2004; Mulvey et al., 2001). Within our laboratory, the typeability and reproducibility of band number, size, position and intensity of both methods was excellent, which supports the validity of the methods regarding their possible use as epidemiological tools. Guidelines by Tenover et al. (1995) state that epidemiologically related strains isolated from a well-defined area during a specific period may possibly be derived from a common source. However, these criteria cannot be applied directly to this study as their basis of comparison is between presumptive outbreak strains and here we have analysed data from random, temporally unrelated samples.

Other studies found similarities between the discriminatory power and reproducibility of both methods. Hahn & Wu (1997) found good reproducibility between rep-PCR and PFGE, with pulsed field being slightly more discriminatory for fingerprinting the Acinetobacter calcoaceticus–Acinetobacter baumannii complex. In a study on transmission of Salmonella, Weigel et al. (2004) found

<table>
<thead>
<tr>
<th>REP-PCR subtype</th>
<th>No. of isolates</th>
<th>PFGE subtype</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>001</td>
<td>18 (9)</td>
<td>A</td>
<td>1 (2)</td>
</tr>
<tr>
<td>002</td>
<td>2 (1)</td>
<td>B</td>
<td>2 (4)</td>
</tr>
<tr>
<td>003</td>
<td>94 (47)</td>
<td>C</td>
<td>11 (21)</td>
</tr>
<tr>
<td>004</td>
<td>24 (12)</td>
<td>D</td>
<td>2 (4)</td>
</tr>
<tr>
<td>005</td>
<td>57 (28.5)</td>
<td>E</td>
<td>22 (42)</td>
</tr>
<tr>
<td>006</td>
<td>1 (0.5)</td>
<td>F</td>
<td>10 (19)</td>
</tr>
<tr>
<td>007</td>
<td>2 (1)</td>
<td>G</td>
<td>4 (8)</td>
</tr>
<tr>
<td>008</td>
<td>2 (1)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
<td>Total</td>
<td>52</td>
</tr>
</tbody>
</table>

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Fig. 1. Banding patterns of REP-PCR subtypes 001–008 (lanes 2–9). Lanes 1 and 10, 2 kb ladder.

Table 1. Comparison of REP-PCR and corresponding PFGE subtypes

Figures in parentheses indicate percentage of total number of isolates tested.
that PFGE and rep-PCR gave similar but not identical classifications and similar conclusions about transmission patterns.

Initial results suggest that REP-PCR may be the optimal method for the routine subtyping of *C. difficile*. Although both methods are highly reproducible and easy to interpret, REP-PCR is easier to perform, has a higher throughput and is less time-consuming than PFGE. It also proves to be slightly more discriminatory over the 52 isolates subtyped by both methods, identifying eight subtypes where PFGE identified seven.

Mapping the distribution of 192 UK rep-types showed regional variations in subtypes distributed throughout the country (Fig. 2). REP-PCR subtype 001 was located in a tight cluster in Northwest England and North Wales with only one exception occurring in Dorchester. Further testing may better identify the nature of the clustering and any sampling bias from this study.

The six remaining UK subtypes occur widely through the country and do not, on initial examination, appear to cluster regionally, showing widespread distribution of all subtypes of *C. difficile* ribotype 001 in hospitals throughout the UK.

Of the 67 isolates from the hospital investigated in the single-centre study, all those referred between 1992 and 1994 (27/67) belonged to REP-PCR subtype 005. Five isolates referred in 1995 typed as REP-PCR subtype 003 and one as REP-PCR subtype 004. Of the isolates received during 1996, 92 % (22/24) were REP-PCR subtype 003 and 8 % were REP-PCR subtype 005. Thus this study revealed a change in predominant subtype in a hospital that occurred between 1994 and 1996 and is an example of how this advanced method can enhance our understanding of local changes in epidemiology.

The isolates sampled for this study were selected from diverse UK regions to maximize the recognition of possible subtypes. Whilst the data are interesting to examine and map, further analyses are required to fully understand the epidemiology of the subtypes. Although both methods have similar discriminatory power and are both highly reproducible, REP-PCR is a more robust method for routine subtyping.

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