Typing of *Clostridium difficile*

J. S. Brazier

Anaerobe Reference Unit, Public Health Laboratory, University Hospital of Wales, Heath Park, Cardiff, UK

Clostridium difficile is primarily recognised as a nosocomially acquired pathogen manifesting in gastrointestinal disease subsequent to the patient receiving broad-spectrum antibiotics. Infection can be sporadic, but outbreaks commonly occur within a ward or hospital as a result of cross-infection. Since the 1980s, the epidemiology of *C. difficile* disease has been studied by the application of many different typing or fingerprinting methods; these, and the lessons learned, are reviewed herein.

Keywords *Clostridium difficile*, typing

Clin Microbiol Infect 2001; 7: 428–431

PHENOTYPIC TYPING METHODS

Early methods of typing *Clostridium difficile* were, of necessity, based on phenotypic properties such as antibiograms. In one of the first documented outbreak investigations, Burdon et al. [1] found a common resistance pattern to three antibiotics in isolates from cases on a surgical ward that were distinct from isolates in the rest of the hospital. However, this approach is at best only rudimentary, and a more detailed approach was tried by Wüst et al. [2] who combined plasmid analysis, soluble protein polyacrylamide gel electrophoresis (PAGE), immunoelectrophoresis of extracellular antigens and antibiograms to 16 isolates from related cases of *C. difficile* infection. Using these methods they showed that 12 of the 16 strains were indistinguishable, providing strong evidence that cross-infection had taken place. Sell et al. [3] used a combination of bacteriocin and bacteriophage typing methods, with a high percentage of strains being non-typeable. Immuno-chemical fingerprinting of EDTA-treated cell extracts of *C. difficile* was evaluated [4], and Nakamura et al. [5] were the first investigators to use serum agglutination as a typing method by raising three antisera against *C. difficile*. This method could differentiate four distinct serovars among 79 isolates from healthy carriers. Delmée's group [6] improved this method and developed a serotyping scheme that could recognise 19 distinct sero-groups. This method is frequently used as the standard by which other typing methods are compared.

These early typing methods were ostensibly developed to understand the epidemiology of *C. difficile* infection at a local level. Many of these investigations found evidence that a single type was responsible for a number of cases within their hospital, thus confirming that *C. difficile* disease could be a cross-infection problem. It soon became apparent, however, that whilst these methods were adequate for local use, there was a need for typing schemes that could be applied to further our understanding of the epidemiology of *C. difficile* disease on a wider scale. To facilitate this, comparisons between typing schemes were performed, and Mulligan et al. [7] found good correlation between the types recognised by plasmid profiling, serotyping, PAGE of cell surface antigens and immunoblotting. Sodium dodecyl sulfate (SDS)-PAGE of whole-cell proteins was applied to 79 isolates in an outbreak investigation and this method yielded a maximum of approximately 40 bands ranging in size from 18 to 100 kilo-daltons (kDa). This investigation showed 60 of the 79 isolates to be indistinguishable. SDS-PAGE of EDTA-extracted cell surface antigens was compared to serogrouping by Ogunsola et al. [8], analysing 61 isolates. This method yielded bands of between 30 and 67 kDa and split their 79 isolates into 17 groups, which generally correlated well with the results of serogrouping, and could in fact, differentiate between some members of the same serogroup. The whole-cell fingerprinting method of pyrolysis mass spectrometry (PMS) has been successfully used as a means of investigating putative *C. difficile* outbreaks [9]. This method has the advantage that it can cope with a large throughput of strains and has a high degree of discrimination. Its disadvantages, however, are the initial cost of the equipment and its inability to assign a permanent type to a strain.

MOLECULAR TYPING METHODS

Molecular typing methods are generally regarded as superior to phenotypic methods in terms of the stability of marker expression and providing greater levels of typeability, and a number of molecular methods have been applied to *C. difficile*. Plasmid profiling proved largely unsuccessful due to the sparse distribution of these extra-chromosomal genetic elements within the species. However, analysis of chromosomal DNA of *C. difficile* was tried by Kuijper et al. [10] who used whole cell DNA restriction endonuclease analysis (REA) using *Hind*III in an

Corresponding author and reprint requests: J. S. Brazier, Public Health Laboratory, University Hospital of Wales, Cardiff CF14 4XW, UK

investigation which demonstrated cross-infection between two patients in the same room. REA is a highly discriminatory and reproducible method; it is, however, a technically demanding procedure and is very labor-intensive, especially for large numbers of isolates. Restriction fragment length polymorphism (RFLP) is an alternative genotypic method that involves initial REA digestion followed by gel electrophoresis and Southern blotting with selected labeled nucleic acid probes to highlight specific restriction site heterogeneity. RFLP, however, is also a very labor-intensive method and REA/RFLP methods have generally been superseded by methods based on the polymerase chain reaction (PCR).

Arbitrarily primed PCR (AP-PCR) is a genotypic method that permits the detection of polymorphisms within the target genome without prior knowledge of the target nucleotide sequence. A closely related method called random amplified polymorphic DNA (RAPD) commonly uses two oligonucleotide primers which are short in length (c.10 bp) and also of arbitrary sequence. Barbut et al. [11] evaluated a RAPD method using two 10-bp primers in an investigation of antiobiotic-associated diarrhea (AAD) in AIDS patients. The same PCR profiles were found in 25 isolates from 15 patients, suggesting infection with the same strain.

PCR ribotyping uses specific primers complementary to sites within the RNA operon and was first applied to *C. difficile* by Gurtler [12] who targeted the amplification process at the spacer region between the 16S and 23S rRNA regions. *C. difficile* was shown to possess multiple copies of the rRNA genes, which not only varied in number between strains but also in size between different copies on the same genome. This approach was simplified by Cartwright et al. [13] who applied it to 102 isolates obtained from 73 symptomatic patients. Using the same primers as Gurtler, their PCR fragments of similar size range could be separated by straightforward agarose gel electrophoresis instead of denaturing PAGE gels. Furthermore, they demonstrated that the banding patterns were not affected by the quantity of DNA used in the reaction (a problem associated with AP-PCR and RAPD methods), and that the PCR ribotype marker was stable and its expression reproducible. This approach was adapted for routine use by O'Neill et al. [14] who improved the methodology even more by greatly simplifying the DNA extraction method. Using modified primers to the 16S–23S spacer region, this method produced amplicons ranging from 250 to 600 bp in length that could be separated by straightforward agarose gel electrophoresis. The discriminatory power was compared to Delmée's serogroups and gave different banding patterns for each of the 19 serogroups. This method has been used routinely by the UK Anaerobe Reference Unit in Cardiff, which has provided a *C. difficile* typing service for the UK since 1995. From over 3000 strains from all sources examined, a library consisting of 116 distinct ribotypes has been constructed [15] (Figure 1).

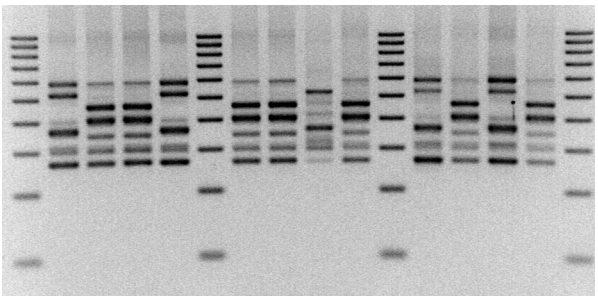

Figure 1 PCR ribotyping gel of *C. difficile* strains (lanes 1, 6, 11 and 16, 100 bp interval ladders).

Pulsed field gel electrophoresis (PFGE) allows the whole genome to be analyzed after digestion with rare cutting restriction endonucleases, such as *Sma*I, *Ksp*I, *Sac*II or *Nru*I, which produce up to 10 fragment length polymorphisms per strain. PFGE has been applied successfully to many different genera and was used to investigate 22 isolates of *C. difficile* from an outbreak in an elderly care facility and 30 epidemiologically unrelated isolates [16]. PCR ribotyping was deemed more discriminatory than AP-PCR and PFGE methods in a study [17]. The authors also highlighted the lack of reproducibility of AP-PCR methods, as discrepancies were noted using the same primers in different laboratories. Whilst PFGE is very discriminatory, disadvantages include the initial cost of the equipment, the slowness of the electrophoresis procedure and its complexity. Bidet et al. [18] compared all three methods and concluded that PCR ribotyping, although marginally less discriminatory than PFGE, offered the best combination of advantages. Spigaglia et al. [19] also found good correlation between PFGE and PCR riboyping, but experienced eight isolates that were non-typeable by PFGE. Many workers have also noted that some strains are repeatedly untypeable by PFGE due to degradation of the extracted DNA. Studies have shown that these PFGE-untypeable strains belong to serogroup G, which corresponds to PCR ribotype 1 in the library of Stubbs et al. [15]. The toxinotyping method developed by Rupnik described 11 toxinotypes and has been compared to PCR ribotyping [20]. Good correlation between the methods was noted and, whilst applying toxinotyping to each type in the PCR ribotype library, five novel toxinotypes were discovered and given ribotypes that had consistent changes in their toxin genes. A recently described alternative PCR target for typing purposes was the flagellin gene *flicC*, described by Tasteyre et al. [21]; *flicC* could discriminate nine different RFLP patterns in a study of 47 isolates.

All typing methods have certain advantages and disadvantages, but their ultimate contribution to knowledge is dictated by their performance according to the criteria listed by Struelens; namely, typeability, reproducibility, stability, discriminatory power and epidemiological concordance [22]. It

should also have technical advantages such as ease of performance, relative low cost and high throughput. In due course, as new methods come and go, one method will probably emerge as being the most suitable.

CONTRIBUTIONS TO KNOWLEDGE BY *C. DIFFICILE* TYPING STUDIES

Since typing methods were applied to *C. difficile*, a picture of our understanding of the epidemiology of *C. difficile* disease on national and international scales has begun to emerge. An international typing study [23] involving seven groups of workers from the UK, Belgium, Australia and the USA was set up and participants were asked to submit their type strains as delineated by their own methods, which included radio-PAGE, immunoblotting, REA, serogrouping, RFLP, PCR ribotyping and AP-PCR. These were checked, blind coded and, together with some wild-type isolates, 100 strains were distributed to each group. Each group typed the set by their own method and submitted their results back to the study coordinator. The preliminary findings of the study were revealing. Many of the groups encountered new strains not previously recognised by their own typing methods, suggesting that there are more types of *C. difficile* in existence than was previously appreciated by each group acting individually. There was complete correlation between the results of the three typing schemes that were based either directly or indirectly on cell surface proteins. This study also revealed that certain types were common to each typing method, indicating distribution of the same types in hospitals in the UK, Belgium, USA and Australia.

Relatively little is known about the national distribution of strains of *C. difficile* circulating the hospitals in individual countries. Probably the most comprehensive national surveillance data have come from the UK Anaerobe Reference Unit in Cardiff, where over 2000 patient stool isolates submitted from 58 UK hospitals have yielded some interesting statistics. In total, 54 different PCR ribotypes have been identified from hospital patients, but just 16 types make up 90% of all referrals, and one particular PCR ribotype, Type 1, accounts for 58% of the total of all hospital patient isolates. The next most common strain, PCR ribotype 106, accounts for just 7%, although this strain has spread from its origins in the midlands to London and the southeast of England in the last few years. PCR Type 1 appears to be endemic in almost all of the hospitals surveyed and is associated with both acute and prolonged outbreaks. It was PCR Type 1 that was responsible for the most publicised outbreak in the UK involving 175 patients and 17 deaths in a hospital in the northwest of England [24]. On an international scale, Brazier's study [23] revealed that PCR Type 1 corresponds to Delmée's serogroup G, and this strain is also causing problems in the USA. PCR Type 1 was found to be the same as strain D1 described by Samore et al. [25], who found this was the most common strain isolated from environmental sources, personnel hand carriage and symptomatic patients in an American East Coast tertiary referral hospital. This same type has also predominated in a series of 59 isolates from elderly male patients in a hospital in California (M. E. Mulligan, personal communication). Research is currently underway in Cardiff applying the whole genome typing method, amplified fragment length polymorphism (AFLP), to *C. difficile* PCR ribotype 1, to determine if it can be subtyped. The prevalence of this strain in the UK is in contrast to findings in some other European countries. Delmée's group reported that serogroup C was most commonly implicated in outbreaks in Belgium [26]. This serogroup corresponds to PCR Type 12, which accounts for only 2.6% of typed hospital isolates in England and Wales. A multicentre study of 11 hospitals in France [27] found serogroups C, D, G and H were the most common strains, with serogroup H predominant (accounting for 21%), and that serogroup C was most often associated with antibiotic treatment and diarrhea. While most studies have shown that a cluster of cases of *C. difficile* infection was due to a single strain, others have demonstrated that clusters of cases have been due to unrelated strains. These sporadic cases demonstrate that not all cases of *C. difficile* infection are due to cross-infection and most probably represent the diverse strains brought in from the community.

It is also known that serogroup F produces toxin B but not toxin A, and corresponds mainly to PCR Type 17. Data from strains received for typing in England and Wales indicate that toxin A-negative/B-positive strains have been detected in 10 UK hospitals [28]. They account for just under 3% of the total hospital isolates examined; although in one particular hospital they accounted for 10% of the total isolates submitted for typing. It is possible that these strains are not being detected because of the common use of diagnostic kits that detect toxin A only, and therefore it may be far more prevalent than we currently appreciate.

Strains originating from General Practice patients and controls in England show a different distribution of PCR ribotypes compared to those found in English hospital patients. The most predominant strain in a community-based study that yielded 390 isolates was PCR Type 10, which is non-toxigenic and accounted for 15.9% of isolates. PCR Type 1, which accounts for 58% of the hospital patient isolates, made up only 7.4% of the community patient isolates. Compared to the overwhelming predominance of one strain in UK hospitals, the profile of types in the community was far more even, with PCR ribotypes 10, 20 and 14 the most common, accounting for 15.9%, 11.8% and 8.7%, respectively. This indicates that certain strains seem to proliferate in hospitals and may even be selected for by local environmental pressures in the hospital ward.

The recently formed ESCMID Study Group on *Clostridium difficile* (ESGCD) has been established to focus on the problem

of *C. difficile* infection from a European perspective. No doubt this, and other typing studies of *C. difficile*, will play a key role in our ongoing attempts to understand the global epidemiology of this nosocomial pathogen and its associated disease.

REFERENCES

1. Burdon DW. *Clostridium difficile*: the epidemiology and prevention of hospital-acquired infection. *Infection* 1982; 10: 203–4.
2. Wüst J, Sullivan NM, Hardegger U, Wilkins TD. Investigation of an outbreak of antibiotic-associated colitis by various typing methods. *J Clin Microbiol* 1982; 16: 1096–101.
3. Sell TL, Schaber DR, Fekety FR. Bacteriophage and bacteriocin typing scheme for *Clostridium difficile*. *J Clin Microbiol* 1983; 17: 1148–52.
4. Poxton IR, Byrne MD. Immunochemical analysis of the EDTA-soluble antigens of *Clostridium difficile* and related species. *J Gen Microbiol* 1981; 122: 41–6.
5. Nakamura S, Mikawa M, Nakashio S, Takabatake M, Okada I, Yamakawa K. Isolation of *Clostridium difficile* from feces and the antibody in sera of young and elderly adults. *Microbiol Immun* 1981; 25: 345–51.
6. Delmée M, Homel M, Wauters G. Serogrouping of *Clostridium difficile* strains by slide agglutination. *J Clin Microbiol* 1985; 21: 323–7.
7. Mulligan ME, Peterson LR, Kwok RY, Clabots CR, Gerding DN. Immunoblots and plasmid fingerprints compared with serotyping and polyacrylamide gel electrophoresis for typing *Clostridium difficile*. *J Clin Microbiol* 1988; 26: 41–6.
8. Ogunsola FT, Ryley HC, Duerden BI. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of EDTA-extracted cell-surface protein antigens is a simple and reproducible method for typing *Clostridium difficile*. *Clin Infect Dis* 1995; 20 (Suppl. 2): S327–30.
9. Magee JT, Brazier JS, Hosein IK, Ribeiro CD, Hill DW, Griffiths A. An investigation of a nosocomial outbreak of *Clostridium difficile* by pyrolysis mass spectrometry. *J Med Microbiol* 1993; 39: 345–51.
10. Kuijper EJ, Oudbier JH, Stuifbergen WNHM, Jansz A, Zanen HC. Application of whole-cell DNA restriction endonuclease profiles to the epidemiology of *Clostridium difficile*-induced diarrhea. *J Clin Microbiol* 1987; 25: 751–3.
11. Barbut F, Mario N, Meyohas MC, Binet D, Frottier J, Petit JC. Investigation of a nosocomial outbreak of *Clostridium difficile*-associated diarrhoea among AIDS patients by random amplified polymorphic DNA (RAPD) assay. *J Hospital Infect* 1993; 26: 181–9.
12. Gurtler V. Typing of *Clostridium difficile* strains by PCR-amplification of variable length 16S–23S rDNA spacer regions. *J Gen Microbiol* 1993; 139: 3089–97.
13. Cartwright CP, Stock F, Beekmann SE, Williams EC, Gill VJ. PCR amplification of rRNA intergenic spacer regions as a method for epidemiologic typing of *Clostridium difficile*. *J Clin Microbiol* 1995; 33: 184–7.
14. O'Neill GL, Ogunsola FT, Brazier JS, Duerden BI. Modification of a PCR ribotyping method for application as a routine typing scheme for *Clostridium difficile*. *Anaerobe* 1996; 2: 205–9.
15. Stubbs SLJ, Brazier JS, O'Neill GL, Duerden BI. PCR targeted to the 16S–23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. *J Clin Microbiol* 1999; 37: 461–3.
16. Talon D, Bailly P, Delmée M *et al*. Use of pulsed-field gel electrophoresis for investigation of an outbreak of *Clostridium difficile* infection among geriatric patients. *Eur J Microbiol Infect Dis* 1995; 14: 987–93.
17. Collier MC, Stock F, DeGirolami PC, Samore MH, Cartwright CP. Comparison of PCR-based approaches to molecular analysis of *Clostridium difficile*. *J Clin Microbiol* 1996; 34: 1153–7.
18. Bidet P, Lalande V, Salauze B *et al*. Comparison of PCR ribotyping, arbitrarily primed PCR, and pulsed field gel electrophoresis for typing *Clostridium difficile*. *J Clin Microbiol* 2000; 38: 2484–7.
19. Spigaglia P, Cardines R, Rossi S, Menozzi MG, Mastrantonio P. Molecular typing and long-term comparison of *Clostridium difficile* strains by pulsed field gel electrophoresis and PCR ribotyping. *J Med Microbiol* 2001; 50: 407–14.
20. Rupnik M, Brazier JS, Duerden BI, Grabnar M, Stubbs SLJ. Comparison of toxinotyping and PCR ribotyping of *Clostridium difficile* strains and description of novel toxinotypes. *Microbiology* 2001; 147: 439–47.
21. Tasteyre A, Karjalainen T, Avesani V *et al*. Phenotypic and genotypic diversity of the flagellin gene (*flicC*) among *Clostridium difficile* isolates from different serogroups. *J Clin Microbiol* 2000; 38: 3179–86.
22. Struelens MJ. Molecular epidemiologic typing systems of bacterial pathogens: current issues and perspectives. *Memoranda Institut Oswaldo Cruz* 1998; 93: 581–5.
23. Brazier JS, Mulligan ME, Delmée M, Tabaqchali S. Preliminary findings of the international typing study on *Clostridium difficile*. *Clin Infect Dis* 1997; 25 (Suppl. 2): S199–201.
24. Cartmill TDI, Panigrahi H, Worsley MA, McCann DC, Nice CN, Keith E. Management and control of a large outbreak of diarrhoea due to *Clostridium difficile*. *J Hospital Infect* 1994; 27: 1–15.
25. Samore MH, Vankataraman L, DeGirolami PC, Arbeit RD, Karchmer AW. Clinical and molecular epidemiology of sporadic and clustered cases of nosocomial *Clostridium difficile* diarrhea. *Am J Med* 1996; 100: 32–40.
26. van Dijck P, Avesani V, Delmee M. Genotyping of outbreak-related and sporadic isolates of *Clostridium difficile* belonging to serogroup C. *J Clin Microbiol* 1996; 34: 3049–55.
27. Barbut F, Corthier G, Charpak Y, Cerf M, Monteil H, Fosse T. Prevalence and pathogenicity of *Clostridium difficile* in hospitalized patients. *Arch Intern Med* 1996; 16: 1449–54.
28. Brazier JS, Stubbs SLJ, Duerden BI. Prevalence of toxin A-negative/toxin B-positive *Clostridium difficile* strains. *J Hospital Infect* 1999; 42: 248–9.