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# Emergence of new PCR ribotypes from the hypervirulent *Clostridium difficile* 027 lineage

Esmeralda Valiente,[1] Lisa F. Dawson,[1] Michelle D. Cairns,[2] Richard A. Stabler[1] and Brendan W. Wren[1]

[1]London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK

[2]Health Protection Agency Collaborating Centre, University College London Hospital, Department of Clinical Microbiology, Windeyer Institute, 46 Cleveland Street, London W1T 4JF, UK

Correspondence
Brendan W. Wren
Brendan.wren@lshtm.ac.uk

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*Clostridium difficile* is the most common cause of antibiotic-associated diarrhoea worldwide. Over the past 10 years, the incidence and severity of disease have increased in North America and Europe due to the emergence of a hypervirulent clone designated PCR ribotype 027. In this study, we sought to identify phenotypic differences among a collection of 26 presumed PCR ribotype 027 strains from the US and the UK isolated between 1988 and 2008 and also re-evaluated the PCR ribotype. We demonstrated that some of the strains typed as BI by restriction endonuclease analysis, and presumed to be PCR ribotype 027, were in fact other PCR ribotypes such as 176, 198 and 244 due to slight variation in banding pattern compared to the 027 strains. The reassigned 176, 198 and 244 ribotype strains were isolated in the US between 2001 and 2004 and appeared to have evolved recently from the 027 lineage. In addition, the UK strains were more motile and more resistant to most of the antibiotics compared to the US counterparts. We conclude that there should be a heightened awareness of newly identified PCR ribotypes such as 176, 198 and 244, and that they may be as problematic as the notorious 027 strains.

## INTRODUCTION

*Clostridium difficile* is a Gram-positive, anaerobic, spore-forming bacillus that is the causative agent of *C. difficile* infection (CDI) (Bartlett, 1994). CDI is often caused after broad-spectrum antimicrobial therapy, which disrupts the barrier effect of the endogenous intestinal microflora allowing *C. difficile* spores to germinate, colonize the gastrointestinal tract and produce toxins, which causes tissue damage (Just *et al.*, 1995, 2001). Antibiotic resistance is likely to be important in infection, as it would provide *C. difficile* a competitive growth advantage in the gut of patients after antibiotic treatment (Delaney *et al.*, 2007). Colonization is also an important step in *C. difficile* pathogenesis, which involves various determinants including surface-layer proteins, adhesins (P47, Cwp66 and Fbp68) (Calabi & Fairweather, 2002; Hennequin *et al.*, 2003; Waligora *et al.*, 2001) and flagella, which have been implicated in adherence of *C. difficile* to caecal mucus in axenic mice (Tasteyre *et al.*, 2001). Flagella have multiple roles in virulence of other enteric pathogens including motility through the viscous intestinal mucosa, chemotaxis, protein secretion and interaction with the innate immune system (Feuillet *et al.*, 2006; Lee *et al.*, 1986; Milton *et al.*, 1996; Moulton & Montie, 1979; Pruckler *et al.*, 1995).

In the last few years, the appearance of highly virulent and epidemic *C. difficile* strains has significantly changed the epidemiology of CDI in North America and Europe (Kuijper *et al.*, 2007; Pépin *et al.*, 2005). This is largely due to the emergence of a clonal lineage referred to using different typing methods as BI [restriction endonuclease analysis (REA)], NAP1 (PFGE), 027 (PCR ribotype) (Killgore *et al.*, 2008) and different clusters [multiple-locus variable-number tandem-repeat analysis (MLVA)] (Eckert *et al.*, 2011). The hypervirulent NAP1 types can be subtyped as NAP1, NAP1a or NAP1c variants, whereas there are at least 23 variants of the REA types, BI-1 to BI-23 (Killgore *et al.*, 2008). MLVA can subtype in different clusters (Eckert *et al.*, 2011). All these strains are invariably classified as toxinotype III by toxinotyping (Rupnik *et al.*, 1998).

The first documented PCR ribotype 027 was described as an isolate from a Parisian hospital in 1985 (Popoff *et al.*, 1988). It was occasionally isolated in the 1990s in the US until major outbreaks of 027-related CDI emerged in 2003 (Morris *et al.*, 2002; Redelings *et al.*, 2007; Ricciardi *et al.*, 2007). There has been a threefold increase in CDI in the elderly, largely as a result of the emergence of the 027 strains during 2000–2005 (Jagai & Naumova, 2009). In the UK, major outbreaks emerged in 2006 where the PCR ribotype 027 was identified. The proportion of 027 strains

Abbreviations: CDI, *Clostridium difficile* infection; MLVA, multiple-locus variable-number tandem-repeat analysis; REA, restriction endonuclease analysis.



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isolated in UK hospitals rose sharply from 25.9 % to 41.3 % between 2005 and 2008 (Brazier *et al.*, 2008). Patients infected with 027 strains have more severe diarrhoea, higher mortality and more recurrences (Goorhuis *et al.*, 2007; Hubert *et al.*, 2007; Loo *et al.*, 2005; Mooney, 2007; Redelings *et al.*, 2007).

A whole genome comparison method using microarrays (comparative phylogenomics) demonstrated that most NAP1/BI/027 isolates formed a single clonal lineage, termed the hypervirulent clade (Griffiths *et al.*, 2010; He *et al.*, 2010; Sebaihia *et al.*, 2006). However, there were exceptions: a single BI-9 (NAP1c) clustered outside the clonal lineage (He *et al.*, 2010; Stabler *et al.*, 2006), identified later as PCR ribotype 001 (He *et al.*, 2010), and BI-14 (NAP1) was an outlier to the hypervirulent clade (Stabler *et al.*, 2006). Interestingly, a previous report showed that not all NAP1 isolates are PCR ribotype 027. For example, strain CA10 was NAP1, but PCR ribotype 019 (Killgore *et al.*, 2008). MLVA has been used to discriminate between isolates with identical PCR ribotypes belonging to types 001, 017 and 027 (van den Berg *et al.*, 2007). Given that many of the PCR-ribotyped *C. difficile* isolates in the US and UK are 027, it is important to develop more accurate methods to distinguish between these highly virulent strains. Recently, we fully sequenced the genomes of two 027 isolates, an historic strain (CD196, the original strain isolated in Paris in 1985) and a modern strain (R20291, the index 027 strain isolated in the UK in 2006), which revealed some genetic differences between them (Stabler *et al.*, 2009). More recently, Nyč *et al.* (2011) reported an outbreak of CDI in the Czech Republic in which the strains were PCR ribotype 176 and closely related to 027 strains. In this study, we determined phenotypic differences in 26 presumed 027 strains from the US and the UK, isolated between1988 and 2008, and re-evaluated their PCR ribotype. We show that the UK and US 027 strains have distinct motility and antibiotic resistance profiles and demonstrate that some strains previously assumed to be 027 are different PCR ribotypes such as 176, 198 and 244.

## METHODS

The 26 human clinical isolates examined in this study are summarized in Table 1. These include BI-1 to BI-15 (provided by Dale Gerding, Hines VA Hospital, USA) and ten PCR ribotype 027 strains, 027-01 to 027-10 (provided by Derek J. Brown, Glasgow, Scotland). In addition, M120 (provided by Denise Drudy, University College, Dublin) and R20291 (provided by Jon Brazier, Cardiff, Wales) were used as controls. *C. difficile* was routinely grown on Brazier's CCEY agar (BioConnections) containing 4 % egg yolk, *C. difficile* supplement (BioConnections) and

**Table 1.** Origin and source of *C. difficile* isolates (1998–2008)

| Code | Isolate reference number | REA type | Date isolated/received | City, state/province | Ribotype |
|---|---|---|---|---|---|
| BI-1 | 1675 | BI-1 | 26/02/1988 | Minneapolis, MN | 027 |
| BI-2 | 4272 | BI-2 | 14/01/1991 | Tucson, AZ | 027 |
| BI-3 | 4233 | BI-3 | 14/12/1990 | Minneapolis, MN | 027 |
| BI-4 | 5325 | BI-4 | 10/02/1993 | Minneapolis, MN | 027 |
| BI-5 | 5604 | BI-5 | 25/08/1995 | Albany, NY | 027 |
| BI-11 | 6296 | BI-11 | 10/08/2001 | Pittsburgh, PA | 198 |
| BI-10 | 6289 | BI-10 | 10/08/2001 | Pittsburgh, PA | 027 |
| BI-6 | 6336 | BI-6 | 20/05/2003 | Portland, OR | 176 |
| BI-7 | 6335 | BI-7 | 20/05/2003 | Portland, OR | 027 |
| BI-8 | 6367 | BI-8 | 22/01/2004 | Portland, ME | 027 |
| BI-6p | 6413 | BI-6p | 09/09/2004 | Atlanta, GA | 027 |
| BI-6p2 | 6431 | BI-6p2 | 09/09/2004 | New Jersey | 027 |
| BI-12 | 6425 | BI-12 | 09/09/2004 | Camp Hill, PA | 027 |
| BI-13 | 6430 | BI-13 | 09/09/2004 | New Jersey | 027 |
| BI-14 | 6432 | BI-14 | 09/09/2004 | New Jersey | 244 |
| BI-15 | 6436 | BI-15 | 09/09/2004 | New Jersey | 027 |
| R20291 | R20291 | | 2006 | Aylesbury, England | 027 |
| 027-01 | 20070031 | | 13/12/2007 | Dundee, Scotland | 027 |
| 027-02 | 20070036 | | 14/12/2007 | Glasgow, Scotland | 027 |
| 027-03 | 20080090 | | 03/04/2008 | Aberdeen, Scotland | 027 |
| 027-04 | 20080107 | | 07/04/2008 | Dumfries, Scotland | 027 |
| 027-05 | 20080195 | | 22/05/2008 | Ayshire, Scotland | 027 |
| 027-06 | 20080238 | | 05/06/2008 | Dumbarton, Scotland | 027 |
| 027-07 | 20080323 | | 26/06/2008 | Edinburgh, Scotland | 027 |
| 027-08 | 20080533 | | 06/08/2008 | Dumbarton, Scotland | 027 |
| 027-09 | 20080684 | | 18/09/2008 | Inverness, Scotland | 027 |
| 027-10 | 20080783 | | 22/10/2008 | Glasgow, Scotland | 027 |
| M120 | M120 | | 2007 | Ireland | 078 |


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1 % defibrinated horse blood (TCS Biosciences), in brain heart infusion (BHI) broth (Oxoid) supplemented with 2.5 % L-cysteine (Sigma Aldrich) and *C. difficile* supplement (Fluka) and on blood agar (agar base; Oxoid) supplemented with 7 % defibrinated horse blood (TCS Biosciences). All cultures were grown from glycerol stocks in an anaerobic atmosphere (10 % $CO_2$, 10 % $H_2$, 80 % $N_2$) at 37 °C.

**PCR ribotyping.** PCR ribotyping was performed at least in duplicate. Briefly, bacteria were harvested from 48 h anaerobic cultures on blood agar. Cells were resuspended into a 5 % (w/v) solution of Chelex-100 (Bio-Rad) and heated to 100 °C for 12 min. The suspension was separated by centrifugation (13 000 ***g*** for 12 min) and the supernatant (10 µl) was added to a 100 µl PCR mixture containing 25 µM each primer (P3, 5′-CTGGGGTGAAGTCGTAACAAGG-3′; and P5, 5′-GCGCCCTTTGTAGCTTGACC-3′), 2.5 units Qiagen HotStar *Taq* DNA polymerase per reaction, 0.4 mM dNTPs and 3.75 mM $MgCl_2$ per reaction. The reaction mixture was subjected to 30 cycles of 9 °C for 1 min, 92 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min. This was followed by 95 °C for 1 min, 55 °C for 45 s and 72 °C for 5 min. The PCR products were concentrated to 40 µl by heating at 75 °C. Electrophoresis was done at 100 mA in 3 % pre-cast Bio-Rad 0.5 % Tris/acetate-EDTA (TAE) agarose gels containing ethidium bromide for 3.5 h at room temperature using pre-chilled TAE buffer. Banding patterns were analysed using GelCompar software (Applied Maths). R20291 was used as a PCR ribotype 027 control.

**Antibiotic susceptibility test.** A range of antibiotics were used to determine the relative susceptibility to clindamycin, erythromycin, chloramphenicol, tetracycline and fluoroquinolones (moxifloxacin, gatifloxacin and levofloxacin). Strain *C. difficile* R20291 with known MICs (Drudy *et al.*, 2006; Stabler *et al.*, 2009) was used as a control. The interpretation of MIC results was based on the recommendations given by the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2008) for all antibiotics used in the present study (Table 2).

Antibiotic susceptibility was determined using the Etest method in all the cases, and the broth dilution method was also used to confirm some of the Etest results. In this case antibiotics were supplied by Sigma-Aldrich.

For the Etest method, a bacterial suspension in BHI to approximately $OD_{600}$=0.5 (McFarland standard no. 3) was plated on blood agar and allowed to dry for 15–30 min. Etest strips (AB Biodisk) were placed onto agar surfaces. Agar plates were incubated anaerobically at 37 °C for a further 24 h, and MICs were determined following the manufacturer's instructions. These were performed in triplicate and R20291 was used as a control (Stabler *et al.*, 2009).

**Table 2.** Antibiotic breakpoints for anaerobes from Clinical and Laboratory Standards Institute guidelines (CLSI, 2008)

| Antibiotics | Susceptible (µg ml$^{-1}$) | Resistant (µg ml$^{-1}$) |
|---|---|---|
| Clindamycin | ⩽2 | ⩾8 |
| Chloramphenicol | ⩽8 | ⩾32 |
| Tetracycline | ⩽8 | ⩾32 |
| Moxifloxacin | ⩽2 | ⩾8 |
| Levofloxacin* | ⩽2 | ⩾8 |
| Gatifloxacin | ⩽2 | ⩾8 |

*Levofloxacin breakpoints could be ⩽2 µg ml$^{-1}$ (susceptible) and ⩾8 µg ml$^{-1}$ (resistant) and erythromycin breakpoints could be ⩽0.5 µg ml$^{-1}$ (susceptible) and ⩾32 µg ml$^{-1}$ (resistant), as no standard has been defined by the CLSI for anaerobes.

In the broth dilution method, bacteria were grown in BHI broth until an $OD_{600}$ of 0.3. Antibiotics were dissolved according to the manufacturer's instructions (Sigma) and tested at a range of 1–128 µg ml$^{-1}$. The MIC was taken as the lowest concentration to inhibit completely visible growth after 24 h growth in an anaerobic chamber at 37 °C. The final MICs were calculated as the mean among the three performed replicates.

**Motility assay.** Motility assay was performed according to the Stabler *et al.* (2009) protocol. Briefly, *C. difficile* cultures were grown anaerobically for 1–2 days on BHI agar. BHI broth plus 0.05 % agar was poured into 30 ml glass vials and placed into an anaerobic chamber. Three single colonies were picked with a loop, inoculated into the top 2–5 mm of BHI agar in the glass vial and left overnight in the anaerobic chamber. The vials were then removed from the chamber and photographed to record the motility. The maximum stalactite length was taken as a measure for motility. The length of those stalactite projections was scored as: <1 cm, non-motile strains; 1–2 cm, motile strain; and >2 cm, highly motile strain. M120 is a non-motile PCR ribotype 078 strain and R20291 is a motile PCR ribotype 027 strain that were used as negative and positive controls, respectively. Experiments were performed in triplicate.

**Autoagglutination assay.** An autoagglutination assay was performed according to Stabler *et al.* (2009). *C. difficile* strains were grown on BHI agar for 1–2 days and then inoculated into pre-reduced 1× PBS to an $OD_{600}$ of 1.0 (±0.1). Then, 2 ml in triplicate was added to pre-reduced glass tubes and incubated for 24 h at 37 °C, after which 1 ml was removed from the tube surface to measure the $OD_{600}$. The results were normalized to the starting OD using the equation 100−[(final OD−starting OD)×100] to show the actual autoagglutination percentage. Strain M120 was used as a positive control as this strain shows more than 95 % autoagglutination; R20291 was used as an autoagglutination negative control as it exhibits a low percentage of autoagglutination. Experiments were performed in triplicate.

**Statistical analysis.** Autoagglutination and motility data were analysed by Tukey's multiple-comparison test using Prism software 9 version 4.0 (GraphPad Software). $P<0.05$ was considered statistically significant.

## RESULTS

### PCR ribotyping

The strains examined in this study are summarized in Table 1. The 16 US isolates were from multiple States between 1988 and 2004 and pre-date the UK strains, which include the UK index strain R20291 isolated in England in 2006 and 10 strains isolated from different regions in Scotland between 2007 and 2008.

Twenty-three of the 26 isolates were confirmed as PCR ribotype 027. The other three strains were different PCR ribotypes: BI-6 was PCR ribotype 176, BI-11 was PCR ribotype 198 and BI-14 was PCR ribotype 244 (Table 1). The PCR ribotype banding profile of 027 strains consisted of seven distinct bands (Fig. 1a, b). Interestingly, the PCR ribotype 198 (BI-11) and PCR ribotype 176 (BI-6) patterns showed a high level of similarity to the PCR ribotype 027 pattern, differing by just a single band. However, strain BI-14 (PCR ribotype 244) differed by the absence of a band



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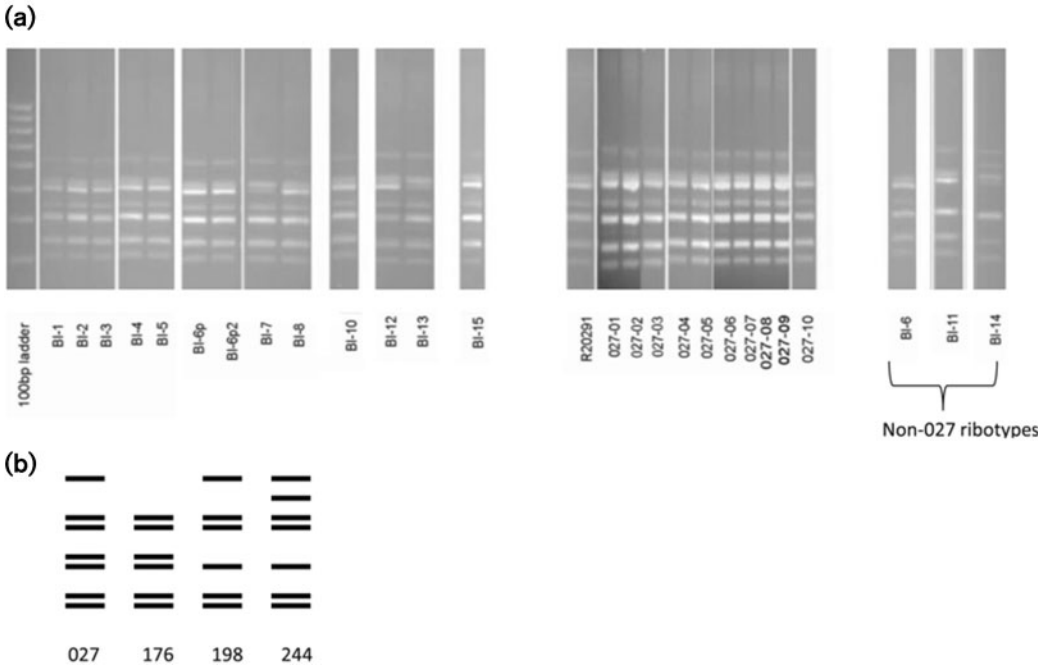


**Fig. 1.** PCR ribotype profiles obtained with *C. difficile* strains. (a) The PCR ribotype banding profile of 027 strains consisted of seven distinct bands (see 027 control). The non-027 strains, PCR ribotype 198 (BI-11) and PCR ribotype 176 (BI-6), differed by just a single band. However, strain BI-14 (PCR ribotype 244) differed by the absence of a band and the addition of a different band. (b) PCR pattern of PCR ribotypes 027, 198, 176 and 244.

and the addition of a different band (Fig. 1a, b). R20291 was used as a control for the 027 PCR ribotype.

### Antibiotic resistance

Antibiotic susceptibility was tested for all isolates (Fig. 2). The majority of PCR ribotype 027 strains exhibited either intermediate resistance (8/26 strains) (4.5–6 μg $ml^{-1}$) or full resistance (15/26 strains) (MIC $\geqslant$128 μg $ml^{-1}$) to clindamycin as well as full resistance to erythromycin (18/26 strains) (MIC $\geqslant$128 μg $ml^{-1}$). The only isolates susceptible to both clindamycin and erythromycin were BI-5 (MIC=2 and 1.75 μg $ml^{-1}$, respectively) and BI-14 (MIC=2 and 1.5 μg $ml^{-1}$, respectively). All UK strains and some US strains were highly resistant to fluoroquinolones (20/26 strains) (MIC $\geqslant$32 μg $ml^{-1}$). However, some US strains were fluoroquinolone-susceptible to moxifloxacin (7/26 strains) (MIC=1 μg $ml^{-1}$), intermediately resistant to levofloxacin (5/26 strains) (MIC=4 μg $ml^{-1}$) and gatifloxacin-susceptible and/or intermediately resistant (3/26 and 4/26 strains, respectively) (Fig. 2).

BI-1 to BI-5 strains, isolated prior to 2003, showed either intermediate resistance (MIC=16 μg $ml^{-1}$) or were fully resistant (MIC $\geqslant$128 μg $ml^{-1}$) to chloramphenicol, whereas the other strains were susceptible to this antibiotic in most cases (18/26 strains) (MIC=5–12 μg $ml^{-1}$). Only BI-7, BI-6p and BI-6p2 were resistant (MIC 18–24 μg $ml^{-1}$) (Fig. 2). All the strains tested were susceptible to tetracycline (MIC $<$0.7 μg $ml^{-1}$). R20291 was used as a control for antibiotic resistance.

### Motility

There was clear and reproducible evidence of motility among 027 isolates (Fig. 3). US strains (16/26 strains) were motile (stalactite length range: 1.6–1.8 cm) whereas UK strains (10/26 strains) were highly motile (stalactite length range: 2.4–3 cm) ($P<0.05$) (Fig. 3). The M120 strain is a non-motile negative control (0.7 cm) and R20291 is a highly motile 027 strain positive control (3 cm) (Fig. 3).

### Autoagglutination

Autoagglutination is often used to demonstrate charge differences on bacterial cells that can be affected by the presence of flagella and how they are modified (Howard *et al.*, 2009). The percentage of autoagglutination was tested for all the isolates. We found a very heterogeneous representation of the autoagglutination phenotype among the strains (Fig. 4).

## DISCUSSION

Rates and severity of CDI have increased alarmingly in recent years and are in part attributable to the emergence and spread of the 027 clonal lineage (Goorhuis *et al.*, 2007; Hubert *et al.*, 2007; Loo *et al.*, 2005; Mooney, 2007). The 26


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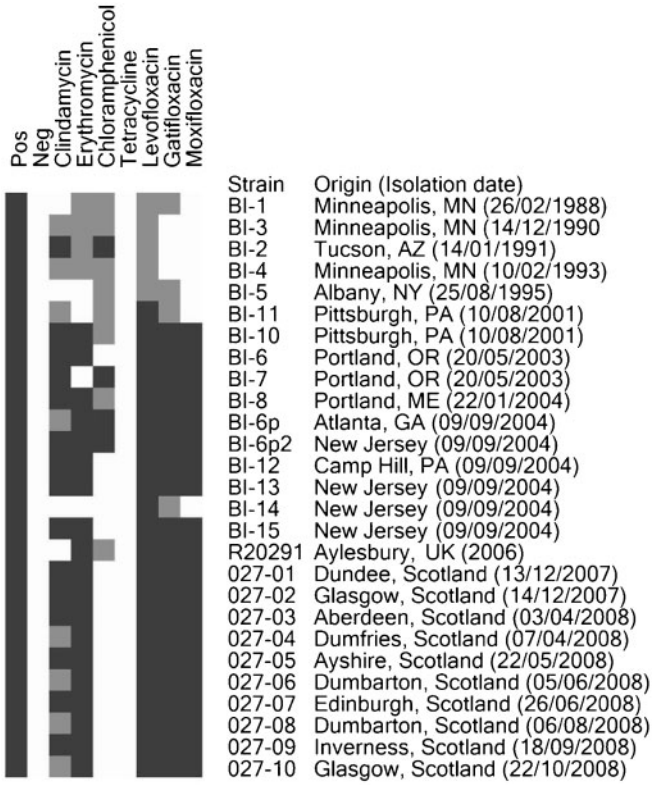


**Fig. 2.** Antibiotic resistance heat map. All 26 strains were tested for their susceptibility to clindamycin, erythromycin, chloramphenicol, tetracycline and fluoroquinolones (levofloxacin, gatifloxacin, moxifloxacin). Black represents resistant, grey represents intermediately resistant and white represents susceptible.

presumed PCR ribotype 027 strains investigated in this study were isolated throughout the UK and the US over a 20-year period. The early strains (1988–2004) were from different States in the US and the later strains (2006–2008) were from the UK (Table 1), which presumably emerged from North America. Interestingly, these isolates are

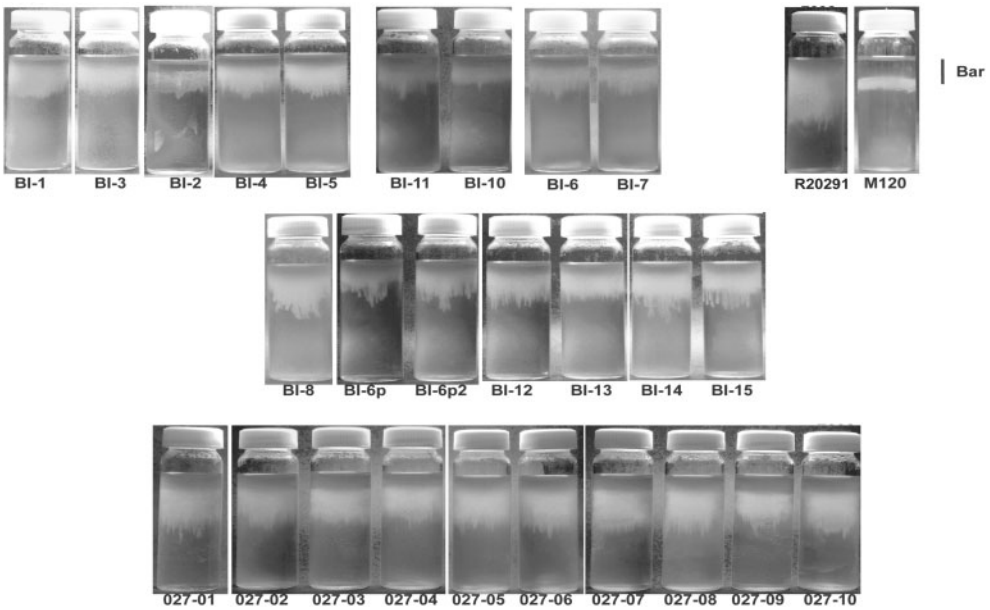


**Fig. 3.** Relative motility assays for *C. difficile* strains. Strains were inoculated into 0.05 % BHI agar and incubated for 24 h in an anaerobic chamber. Motility is visualized as stalactite projections. M120 and R20291 are negative and positive controls, respectively. Bar, 1.2 cm.


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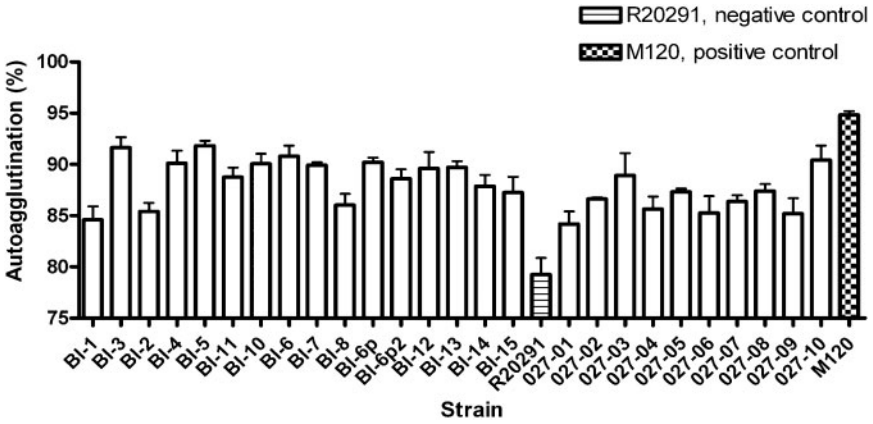


**Fig. 4.** Relative autoagglutination of *C. difficile* strains. *C. difficile* strains were grown on BHI plates for 1–2 days, then inoculated into pre-equilibrated PBS to an $OD_{600}$ of 1.0 (±0.1). These were incubated for 24 h in pre-equilibrated glass tubes, and then the $OD_{600}$ was measured. The percentage autoagglutination was normalized to the starting $OD_{600}$. The bars indicate the percentage of cells autoagglutinating. M120 was used as a positive control.

generally referred to as BI/NAP1/027, with the assumption that BI types and NAP1 types are PCR ribotype 027. However, we have shown that the REA type 'BI' and the PFGE type NAP1 do not always correlate with PCR ribotype 027. PCR ribotype analysis revealed that BI-6, BI-11 and BI-14 were not PCR ribotype 027 as previously presumed. BI-14 was an outlier strain in a previous study (Stabler *et al.*, 2006). In our study, BI-14 had two different bands compared to PCR ribotype 027, being assigned as PCR ribotype 244. However, the similarity of the PCR ribotype banding pattern for BI-11 (PCR ribotype 198) and BI-6 (PCR ribotype 176) was high. Slight band differences between PCR ribotype 176 and presumed PCR ribotype 027 *C. difficile* isolates have also been observed in Austria (A. Indra, personal communication in 2010) and more recently in the Czech Republic and in Poland (Nyč *et al.*, 2011). Despite being different ribotypes, it is clear that the BI-11 (PCR ribotype 198) and BI-6 (PCR ribotype 176) strains are closely related to 027 strains and have previously been placed in the same clade using comparative genome microarray analyses (Stabler *et al.*, 2006). High similarity in PCR ribotyping pattern between 027 and other ribotypes should be monitored, as strains from these ribotypes may be as problematic as the hypervirulent 027 strains.

CDI is frequently linked to treatment with antibiotics which provide an advantage for *C. difficile* survival (Delaney *et al.*, 2007). In this study, we showed that UK strains were resistant to most of the antibiotics tested. However, US strains showed a variable range of antibiotic resistance depending on the antibiotic. It is a reasonable hypothesis that these changes in antibiotic resistance profiles could be linked to the changes in antibiotic prescription policy over time and in different countries. The most noteworthy is the rise in resistance to fluoroquinolones such as levofloxacin, gatifloxacin and moxifloxacin. With few exceptions, antibiotic resistance in *C. difficile* is transposon-mediated: erythromycin resistance (*ermB*) is carried on Tn*5398* (Hussain *et al.*, 2005; Sebaihia *et al.*, 2006) or CTn*CD11* (He *et al.*, 2010), chloramphenicol resistance (*catD*) is carried on Tn*4453* (Lyras *et al.*, 2004) and tetracycline resistance is carried on Tn*5397* (Hussain *et al.*, 2005). Previous studies identified a novel transposon in R20291, CTn-*027*, encoding a chloramphenicol resistance gene (CD*R20291_3461*) in some PCR ribotype 027 strains (Stabler *et al.*, 2009). However, all UK strains with the exception of R20291 (used as a control) and some US strains were chloramphenicol-susceptible with some exceptions, indicating that the transmission of this transposon-mediated antibiotic resistance might not be uniform throughout the 027 lineage. On the other hand, tetracycline resistance (*tetM*), usually carried on Tn*5397*, was absent in all 26 strains tested. Generally, resistance to macrolides (e.g. erythromycin) and lincosamides (e.g. clindamycin) is mediated via the presence of the *ermB* gene and it is more common in 027 isolates (Solomon *et al.*, 2011). Although 14 strains were erythromycin- and clindamycin-resistant, Tn*CD11* carrying *ermB* was only present in five of the BI isolates (BI-6, BI-7, BI-10, BI-13 and BI-15) analysed by whole genome analysis (He *et al.*, 2010). This suggests an alternative mechanism for *ermB* resistance in the 027 lineage. The development of antibiotic resistance in modern 027 strains emphasizes the importance of antibiotic susceptibility testing for the emergence of antibiotic resistance.

The flagella-associated genes in strain 630 (PCR ribotype 012) are found in two loci, F1 and F3, separated by an inter-flagella locus, F2 (Stabler *et al.*, 2009). The level of sequence identity in 027 strains compared to the 630 strain for the F1 and F3 region was high, but there were significant differences in the F2 region (Stabler *et al.*, 2009). This may provide a genetic basis for the motility differences among 027 isolates used in this study, but to date available genetic information on the strains in this study cannot explain the difference in motility between the 027 strains. Modifications in motility


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and antibiotic resistance between the 027 strains may reflect a genetic change with respect to the flagella and glycosylation loci as well as the loss and gain of transposons and the accumulation of mutations over time. Such changes could be beneficial for invasion, adhesion, access to nutrients and general survival and transmissibility of *C. difficile*.

Understanding the evolution of clones such as the PCR ribotype 027 will be important in predicting the early emergence (or disappearance) of highly virulent *C. difficile* strains and represents a current public health imperative. In this study, phenotypic differences between the 26 UK and US strains suggest that the PCR ribotype 027 is genetically variable. It appears that the 176 and 198 ribotypes have evolved recently from the 027 lineage. Thus there should be a heightened awareness that the recently identified PCR ribotypes 176 and 198 may be as problematic as the 027 strains.

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