Molecular characterization and antimicrobial susceptibility patterns of *Clostridium difficile* strains isolated from hospitals in south-east Scotland

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*Clostridium difficile* isolates (*n* = 149) collected in south-east Scotland between August and October 2005 were typed by four different methods and their susceptibility to seven different antibiotics was determined. The aims were to define the types of strain occurring in this region and to determine whether there were any clonal relationships among them with respect to genotype and antibiotic resistance pattern. Ribotyping revealed that 001 was the most common type (*n* = 113, 75.8%), followed by ribotype 106 (12 isolates, 8.1%). The majority of the isolates (96.6%, *n* = 144) were of toxinotype 0, with two toxinotype V isolates and single isolates of toxinotypes I, IV and XIII. PCR and restriction analysis of the *fliC* gene from 147 isolates gave two restriction patterns: 145 of pattern VII and two of pattern I. Binary toxin genes were detected in only three isolates: two isolates of ribotype 126, toxinotype V, and one isolate of ribotype 023, toxinotype IV. S-types showed more variation, with 64.5% (*n* = 40) of the common S-type (4939) and 21% (*n* = 13) of S-type 4741, with six other S-types (one to three isolates each). All ribotype 001 isolates were of the same S-type (4939), with three isolates of other ribotypes being this S-type. No resistance was found to metronidazole or vancomycin, with resistance to tetracycline only found in 4.3% of the isolates. A high proportion of isolates were resistant to clindamycin (62.9%), moxifloxacin, ceftriaxone (both 87.1%) and erythromycin (94.8%). Resistance to three antibiotics (erythromycin, clindamycin and ceftriaxone) was seen in 66 isolates, with erythromycin, ceftriaxone and moxifloxacin resistance seen in 96 isolates. Resistance to all four of these antibiotics was found in 62 isolates and resistance to five (the above plus tetracycline) in one isolate: a ribotype 001, toxinotype 0 strain. Whilst ribotype 001 was the most commonly encountered type, there was no evidence of clonal relationships when all other typing and antibiotic resistance patterns were taken into account.

INTRODUCTION

*Clostridium difficile* is an anaerobic, Gram-positive, spore-forming bacillus. It is commonly associated with a spectrum of disease referred to as *C. difficile*-associated disease (CDAD), which can range from uncomplicated mild diarrhoea to lethal toxic megacolon and possible colon perforation (Johnson & Gerding, 1998). It is considered to be the leading cause of nosocomially acquired diarrhoea in adults and can be responsible for large outbreaks (Kelly & LaMont, 1998). There is a view that the severity of the disease is increasing. The new hypervirulent type (ribotype 027, toxinotype III, pulse-field NAP1) in North America and several European countries has been associated with more severe and fatal cases (McDonald et al., 2005; Kuijper et al., 2006a; Hubert et al., 2007).

As elsewhere, *C. difficile* is rarely cultured in Scotland and laboratory diagnosis depends on the detection of toxins A and/or B in faeces. Several phenotypic and molecular methods have been applied to determine the relatedness of strains of *C. difficile*. All have their advantages and disadvantages. Methods based on whole-genome analysis are more discriminatory, but they are technically demanding and labour-intensive (Brazier, 2001). PCR ribotyping is commonly used in Europe as it has been reported to be
highly discriminative, reproducible, relatively rapid and easy to perform (O’Neill et al., 1996; Stubbs et al., 1999). Toxinotyping is a PCR-RFLP method that depends on changes in the toxin genes and other regions of the pathogenicity locus of \textit{C. difficile}. It has been reported to correlate well with restriction endonuclease analysis, serotyping and PCR ribotyping and it also gives the advantage of determining toxin variant strains (Rupnik et al., 1998; Johnson et al., 2003). Flagellin gene RFLP analysis has been described as an additional typing method that can be used in conjunction with other typing methods (Tasteyre et al., 2000).

An actin-specific ADP-ribosylating binary toxin CDT is produced by some strains of \textit{C. difficile}. Its role in pathogenesis is currently unclear, but its presence has been correlated with severity of disease in some studies (Barbut et al., 2005) and it is present in the 027 hypervirulent strain (McDonald et al., 2005). The prevalence of binary toxin in clinical isolates of \textit{C. difficile} is generally low, with frequencies ranging between approximately 2 and 20\% (Barbut et al., 2005).

The aims of this study were to (i) characterize 149 \textit{C. difficile} isolates from toxin-positive faecal samples collected between August and October 2005 by molecular typing methods, PCR ribotyping, toxinotyping and flagellin gene RFLP analysis and by the S-layer typing method, together with the detection of the binary toxin genes \textit{cdtA} and \textit{cdtB}; and (ii) determine the susceptibility of isolates to seven different antibiotics. The objectives were to show which strains were currently present locally, to determine whether there were any clonal relationships between isolates and to examine the antimicrobial susceptibility profiles of the different types.

**METHODS**

**Bacterial isolates.** \textit{C. difficile} isolates (\textit{n}=149) from toxin-positive faecal samples (determined using a Toxin A + B ELISA kit; TechLab) were collected between August and October 2005 and stored at \(-20^{\circ}\) C. All specimens were from different unselected cases of CDAD in different hospitals in the Edinburgh area (Lothian University Hospitals National Health Service Trust) consisting of acute and long-stay hospitals. They were collected on a purely random basis with no selection for hospital or patient type. During this period, we were not aware of any outbreaks. Stool samples were cultured on Brazier’s cefoxitin/cycloserine/egg yolk agar (LabM) and incubated for 48 h at 37\(^{\circ}\) C in an anaerobic chamber. The isolates were identified by characteristic colony morphology, smell, fluorescence under long-wave UV light and appearance on a Gram film. Subcultures were stored in anaerobic investigation medium containing cooked meat particles for maintenance (Brown et al., 1996). Control strains were NCTC 11223, VPI 10463, 338a (a locally isolated strain of ribotype 01; McCoubrey, 2002), the sequenced strain 630 and a 027 strain from Amsterdam provided by E. Kuijper (Leiden, The Netherlands) (Bio-Rad). After incubating in a boiling bath for 10 min, the cell debris was removed by centrifugation for 2 min at 18 000 g. The supernatant was used as the crude DNA template for PCRs except for toxinotyping.

For toxinotyping, pure DNA isolation was required. DNA was extracted using a Nucleospin Tissue kit (Macherey-Nagel) according to the manufacturer’s instructions.

**PCR ribotyping.** All 149 isolates were typed by PCR ribotyping according to the method described by O’Neill et al. (1996). Specific oligonucleotide primers 5'-CGGGAGGGTGATGTGCTAAAGG-3' (nt 1445–1466 of the 16S rRNA gene) and 5'-GGCCCTTTGTTAGCCTTACC-3' (nt 20–1 of the 23S rRNA gene) complementary to the 3' end of the 16S rRNA gene and the 5' end of the 23S rRNA gene were used to amplify the variable-length intergenic spacer region. The 338a and 027 strains were used as controls for ribotypes 001 and 027. Patterns that were different from these two ribotypes were compared with the library of PCR ribotypes already established at the Anaerobe Reference Unit, Cardiff, UK.

**Toxinotyping.** All 149 isolates were subjected to toxinotyping by the methods developed by Rupnik et al. (1997, 1998). The first 3 kb of \textit{tdcb} (PCR fragment B1) and the 3 kb repetitive region of \textit{tdcb} (PCR fragment A3) were detected and characterized by RFLP. The primers 5'-AGAAAATTTTATGGTTAGATATAGAAA-3' and 5'-CAGATAAGAAGGAAGTACGCTATAG-3' for the B1 fragment and 5'-TATAATGCGACGGATATGTAAGGAC-3' and 5'-TTATGAAACATATATTTAGGATATAC-3' for the A3 fragment were used as described by Rupnik et al. (1997). PCRs were performed in a final volume of 50 \micro liter with a reaction mixture containing 20 mM Tris/ HCl (pH 8.3), 50 mM KCl, 1% W-1, 3 mM MgCl\(_2\), 1 U Taq polymerase (Invitrogen), 200 \micro M each dNTP, 15 pmol each primer and 5 \micro liter template DNA. For amplification of A3 fragments, tetramethylammonium chloride (Sigma) was added to a final concentration of 10\(-4\) M. After initial denaturation at 93\(^{\circ}\) C for 3 min, B1 products were amplified for 30 cycles and A3 products for 35 cycles of annealing and extension at 47\(^{\circ}\) C for 8 min and denaturation at 93\(^{\circ}\) C for 4 s. Final extension was at 47 \(^{\circ}\) C for 10 min. Amplified fragments were visualized on a 1% agarose gel and subjected to restriction enzyme digestion using the restriction enzymes \textit{AccI}, \textit{HincII} (B1) and \textit{EcoRI} (A3). After electrophoresis of the digestion products, the toxinotypes of all tested isolates were determined using the toxinotyping schema described by Rupnik et al. (1997, 1998).

**Detection of binary toxin genes.** The presence of binary toxin genes among all 149 study isolates was detected by PCR as described by Stubbs et al. (2000). Primers designed to amplify the genes encoding the enzymic (\textit{cdta}) and binding (\textit{cdtb}) components of the binary toxin were as follows: CDTA-F, 5'-TGAACCTGGAAAGGGTATG-3'; CDTA-R, 5'-AGGATATTTACTGAGCCATTTTG-3'; CDTB-F, 5'-CTTAAAGGAATATAACTGAG-3'; CDTB-R, 5'-AACGAGTCTTTGCTTACGTC3'. The 027 strain, which is known to produce binary toxin, was used as the positive-control strain. The product sizes for \textit{cdta} and \textit{cdtb} were 375 and 510 bp, respectively.

**PCR-RFLP analysis of the flagellin (\textit{fliC}) gene.** The \textit{fliC} gene of 147 of the study isolates (two were lost) was amplified using the specific primers Nter (5'-ATGAGAGTTAATCAAATGTAGTGCG-3') and Cter (5'-CTATCTATGAATTGTAATAAACCTCC-3') corresponding to the 5'- and 3'-end sequences of the \textit{fliC} gene of \textit{C. difficile} (Tasteyre et al., 2000). Amplification was carried out in a final volume of 50 \micro liter reaction mixture containing 20 mM Tris/HCl (pH 8.3), 50 mM KCl, 2 \micro M MgCl\(_2\), 1 U Taq polymerase (Promega), 0.2 \micro M each dNTP, 1 \micro M each primer and 5 \micro liter template DNA. Initial denaturation was carried out at 94\(^{\circ}\) C for 5 min, followed by
35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. A final step of extension for 10 min at 72 °C was performed. Products of 870 bp were digested with the restriction enzymes HpaI, HindIII and Rsal. Digested products were electrophoresed on a 1.2 % agarose gel to determine their RFLP groups. The restriction enzyme HincII was used for further differentiation between group I and group III flagella types.

**S-layer typing.** The S-layer typing of *Clostridium difficile* isolates was performed as described previously (McCoubrey et al., 2003). Briefly, the isolates were subcultured and S-layer proteins were extracted with 5 M guanidine hydrochloride. The resulting two major and several minor bands were visualized by SDS-PAGE (Invitrogen) with Coomassie staining. Mark 12 molecular mass standards were used as calibrations for the calculation of molecular masses. Banding patterns were compared with the previous types.

**Antibiotic susceptibility testing.** The MICs of 116 of the isolates for six antibiotics were determined using the agar dilution protocol in the NCCLS guidelines (NCCLS, 2001). The antibiotics and concentrations used were as follows: 16–0.125 μg ml⁻¹ for vancomycin, 32–0.5 μg ml⁻¹ for metronidazole, 256–4 μg ml⁻¹ for ceftiraxone, 32–0.5 μg ml⁻¹ for clindamycin, 32–0.5 μg ml⁻¹ for erythromycin and 64–1 μg ml⁻¹ for tetracycline (all from Sigma). The isolates were subcultured from cooked meat broth into pre-reduced thioglycollate medium (Sigma) enriched with 5 % lysed horse blood plus antibiotic of a given concentration using a multipoint inoculator and incubated anaerobically at 37 °C, 5 % NaHCO₃ ml⁻¹ and incubated overnight in an anaerobic chamber at 37 °C. After adjusting the turbidity to a 0.5 McFarland standard, aliquots (1–2 μl) of the cultures were spotted onto Brucella agar (Oxoid) supplemented with haemin, vitamin K₁ and 5 % lysed horse blood plus antibiotic of a given concentration using a multipoint inoculator and incubated anaerobically at 37 °C for 48 h. Control plates were also inoculated and incubated aerobically to check for aerobic growth. Strains NCTC 11223, 338a and 630 were used as control strains as their MICs were known from our previous study (Drummond et al., 2003).

The MICs of the isolates for moxifloxacin were determined by the Etest (AB Biodisk) as we were unable to obtain the pure substance from Bayer. The isolates were grown in pre-reduced thioglycollate medium (Sigma) enriched with 5 μg haemin, 1 μg vitamin K₁ and 1 mg NaHCO₃ ml⁻¹ and incubated overnight in an anaerobic chamber at 37 °C. The Etest was carried out by inoculating the surface of pre-reduced fastidious anaerobe agar (LabM) plates containing vitamin K₁, haemin and 5 % lysed horse blood with a 1 McFarland standard-matched inoculum. The inoculation was performed with cotton-tipped swabs and Etest strips were applied to the agar surface according to the manufacturer’s instructions. Sufficient growth was obtained after 24 h and the ellipse was clearly visible. The end points were read at complete inhibition of all growth, including hazes and isolated colonies. Strains 630 and 027 were used as sensitive and resistant controls, respectively.

Breakpoints of susceptibility for each drug were chosen at the levels listed by the NCCLS. MIC₅₀ and MIC₉₀ values for each isolate were calculated using Microsoft EXCEL.

**RESULTS AND DISCUSSION**

**Molecular typing**

The 149 isolates included in this study were collected over a period of 2 months between August and October 2005. The results of the different typing methods are summarized in Table 1. All of the isolates were typable by the PCR ribotyping method and 15 different ribotype patterns could be discriminated. Ribotype 001 was the most common (n=113, 75.8 %), followed by ribotype 106 with 12 isolates (8.1 %). The other ribotypes identified were 005 and 014 with four isolates each, ribotype 002 with three isolates and ribotypes 013 and 126 with two isolates, whilst the other ribotypes (020, 023, 042, 049, 070, 171) were represented by single isolates. Three isolates belonging to two different ribotypes were not able to be allocated a specific ribotype and may represent new types. No 027 strain was found. However, during the preparation of this manuscript the first case of 027 in Scotland was reported from the Glasgow area. These findings are consistent with other reports from Scotland.

**Table 1. Numbers and types of isolates determined by PCR ribotyping, toxinotyping and fliC restriction analysis**

<table>
<thead>
<tr>
<th>Ribotype (no. detected out of 149)</th>
<th>Different toxinotypes found for each ribotype</th>
<th>Different fliC patterns for each ribotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>I</td>
</tr>
<tr>
<td>001 (113)*</td>
<td>111</td>
<td>1</td>
</tr>
<tr>
<td>106 (12)*</td>
<td>12</td>
<td>–</td>
</tr>
<tr>
<td>005 (4)</td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td>014 (4)</td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td>002 (3)</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>013 (2)</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>126 (2)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>020 (1)</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>023 (1)</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>042 (1)</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>049 (1)</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>070 (1)</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>171 (1)</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>Others (3)</td>
<td>3</td>
<td>–</td>
</tr>
</tbody>
</table>

*One isolate from each of these ribotypes could not be typed by fliC restriction analysis.
the UK prior to the recognition of the 027 strains in several areas of England. PCR ribotype 001 has been reported to be the most common type (55%) among hospitalized patients in the UK (Stubbs et al., 1999). In an earlier study by our group, ribotype 001 was responsible for 78% of CDAD infections locally (McCoubrey et al., 2003). Recently, ribotype 106 has become prominent in England. In the period 1995–2003 it was at similar levels to those found in our study (8%); however, in 2005 it was the predominant strain at 26%, just above 027 strains and surpassing the 001 strains, which were both at 25% (Health Protection Agency, 2006). Prior to the recognition of 027 strains in Europe, particularly in The Netherlands and Belgium, there are only a few reports from other countries in Europe. In a Polish hospital, all environmental isolates and 11 of 31 neonatal isolates were found to belong to ribotype 001 (Martirosian et al., 1995), whereas ribotype 087 accounted for 39% of all isolates in Hungary (Urban et al., 2001). In one report from the Middle East, ribotypes 097 and 078 were reported to be responsible for over one-third of the cases of CDAD in Kuwaiti hospitals (Rotimi et al., 2003).

All of the isolates were subjected to toxinotyping. The B1 and A3 fragments of tcdA and tcdB were amplified as they have been reported to be the most variable fragments and good markers when searching for variant strains (Rupnik, 2001). C. difficile VPI 10463 (which has been defined as toxinotype 0; Rupnik et al., 1998) was used as a reference strain. This toxinotype 0 was observed in the majority of the isolates (96.6%, n = 144). The five remaining isolates were of four different known toxinotypes: toxinotypes I, IV and XIII with one isolate each and toxinotype V with two isolates. No toxinotype III strains were found. There was no previous local information on the prevalence and distribution of different toxinotypes. When compared with the studies from Europe, the rates of variant (not 0) toxinotypes in our study were lower (Rupnik et al., 1998, 2001; Spigaglia & Mastrantonio, 2002). The profiles of the strain collections in these studies were different but non-toxinogenic strains were not included in our study. The prevalence of variant toxinotypes has been reported to be 21.5% among the selected C. difficile isolates from 22 serogroups tested by Rupnik et al. (1998) and was estimated for the Cardiff collection as 8.8% among the toxigenic strains (Rupnik et al., 2001). The percentage of variant strains from Asia has been reported to be 23.5%, whilst 25% of the toxigenic strains from Italy were found to show variation (Spigaglia & Mastrantonio, 2002; Rupnik et al., 2003a). Of the strains from an American hospital, 11.1% belonged to variant toxinotypes. The most frequent variant toxinotypes in two European collections were toxinotypes III, IV and VIII (Rupnik et al., 1998, 2001). Our variant strains were of toxinotypes I, IV, V and XIII. The toxinotype IV strain was of ribotype 023, whilst two toxinotype V strains were of ribotype 126. Toxinotypes I and XIII were of ribotype 001. In our study, among the 15 ribotypes that were determined, all isolates within a ribotype except ribotype 001 belonged to a single toxinotype. This was similar to the findings of Rupnik et al. (2001) where PCR ribotyping and toxinotyping were shown to correlate well. In the case of different toxinotypes within a PCR ribotype, these toxinotype profiles were found to be similar. In our study, only five isolates of toxinotypes other than 0 were found and isolates of ribotype 001 belonged to three different toxinotypes. Toxinotype I and XIII strains have been reported to differ from toxinotype 0 only at the 3′ end of the tcdA gene (Rupnik et al., 1998).

PCR amplification of the fliC gene from 147 isolates produced an 870 bp fragment. Two different restriction profiles were obtained when the amplification products were digested using the enzymes HpaI, HinDI and Rsal. All isolates but two were of restriction pattern VII, the exceptions being restriction pattern I, and were of ribotypes other than 001 (ribotypes 106 and 042) but were of toxinotype 0. Tasteyre et al. (2000) compared PCR-RFLP analysis of the flagellin gene and serogroups in a collection of strains representing all of the 12 known serotypes from widely different geographic areas. They reported that this method could constitute an additional typing method to be used in conjunction with other methods. They found RFLP type VII as the most frequent RFLP type, followed by types I and VIII. RFLP type VII strains were mostly toxin-positive strains, whereas type I strains were either toxin positive or negative. They found that RFLP types II, III, IV, V and VI were uncommon and only associated with single serogroups. Our study is the first to compare ribotyping and toxinotyping with flagellin gene typing. However, only two flagella types were detected (I and VII): type VII strains contained different ribotypes and toxinotypes and type I strains were of different ribotypes but all of toxinotype 0. Thus, a larger number of strains from different ribotypes and toxinotypes are needed to be able to determine the relationships between these typing methods.

All strains (n = 149) were tested for the presence of binary toxin genes (cdtA and cdtB), but only three (2%) harboured these genes. Similar to the number of variant strains encountered above, the presence of binary toxin genes was low compared with other studies. It has been reported that 6.4% of toxigenic isolates of C. difficile referred to the Anaerobe Reference Unit from UK hospitals had both binary toxin genes (Stubbs et al., 2000) and 4.5, 5.8 and 8.6% prevalence of binary toxin-positive strains was detected in Spain, America and Poland, respectively (Geric et al., 2004; Alonso et al., 2005; Pituch et al., 2005). Pituch et al. (2005) found that all binary toxin-positive strains from Poland were of the same toxinotype, type IV, and were all of the same ribotype. In our study, two of the binary toxin-positive isolates were of ribotype 126, toxinotype V, and one isolate belonged to ribotype 023, toxinotype IV. In most studies, it has been shown that only strains belonging to variant toxinotypes that have significant changes in tcdA and tcdB possess binary toxin genes (Stubbs et al., 2000; Rupnik et al., 2003b).
(2003) reported A⁻B⁻ strains with binary toxin genes. We had only toxigenic strains in our study. Our strains of toxinootypes IV and V with changes in both the tcdA and tcdB genes had binary toxin genes, whereas strains of toxinootypes I and XIII with minor differences only in the 3' end of the tcdA gene did not have cdtA or cdtB.

S-layer typing
In the past, we have used S-typing as our primary method for epidemiological studies of C. difficile (McCoubrey et al., 2003). We were interested in correlating the different molecular types with S-type. However, as it is relatively labour-intensive, we selected a sample of only 62 isolates to represent the range of molecular types described above with the result that six different S-types were recognized. Most isolates (64.5 %; n=40) belonged to the common S-type, known as type 4939, named for the molecular masses of the two S-layer peptides. In an earlier paper (McCoubrey et al., 2003), this common S-type, which is that of ribotype 001, was referred to as 5336. We have recently changed our SDS-PAGE system to a commercial system (Invitrogen) employing 10 % gels. With this new method, the molecular masses of the S-layer proteins are found to be different. Most of the others were of S-types 4741 (21 %; n=13) and 4640 and 4938 with three isolates each (4.8 %). Of the remainder, two isolates (3.2 %) were of S-type 4639 and one isolate (1.6 %) was of S-type 4837. All ribotype 001 isolates (n=37) were of the same S-type (4939), whilst three of the isolates from different ribotypes were also of this S-type. All but one of the ribotype 106 isolates (n=11) were of S-type 5242, the other being of the common 4939 type. Ribotypes 002 and 014 also contained different S-types. The toxinotype 0 isolates (n=59) belonged to all six S-types, with the most common being S-type 4939 (n=38). Toxinotype I and XIII isolates, which were also of ribotype 001, were of S-type 4939.

The discriminatory power of different typing methods is an important consideration when selecting which method to use. The usual gold standard of PFGE is generally considered to have a higher degree of discrimination than PCR ribotyping. However, in the past, the typing ability of PCR ribotyping was higher than that of PFGE because DNA degradation occurred as a result of endogenous restriction enzymes in strains from serogroup G, which corresponds to PCR ribotype 001 (Collier et al., 1996; Bidet et al., 2000).

Currently, PCR ribotyping is preferred because of the ease and speed of the technique and because it is reported to be highly discriminatory and reproducible. Toxinotypes are reported to correlate well with the types obtained by two other typing schemes, serogrouping and PFGE typing (Rupnik et al., 1998), whilst toxinotyping and ribotyping methods correlate well. Most strains within a PCR ribotype belonged to a single toxinotype. Strains in toxinotypes I, III, IV, VI and VIII could be differentiated into several PCR ribotypes (Rupnik et al., 2001).

Antibiotic susceptibility testing
A major aim of this study was to determine the current antibiotic susceptibility patterns of the C. difficile isolates in our region and to find out whether there was any relationship between the types and antibiotic susceptibilities. The susceptibility to antibiotics was investigated in a sample of 116 isolates of our collection by determining the MICs for seven antibiotics: metronidazole, vancomycin, erythromycin, clindamycin, ceftriaxone, moxifloxacin and tetracycline. Table 2 shows the ranges of MICs and resistance rates among the isolates for the seven antibiotics used, together with MIC₅₀ and MIC₉₀ values and breakpoints for the antibiotics. All isolates were sensitive to the two agents commonly used to treat CDAD, metronidazole and vancomycin, with a narrow range of MICs.

In our previous study (Drummond et al., 2003), no resistance to metronidazole or vancomycin was reported. MIC ranges and MIC₅₀ and MIC₉₀ values for these antibiotics were similar to those in the present study. However, the number of isolates with an MIC of 4 µg ml⁻¹ for vancomycin increased from 5 out of 186 isolates (2.7 %) in our earlier study to 25 out of 116 isolates (21.6 %) in the present study. Vancomycin and metronidazole are the most common antibiotics used in the treatment of CDAD and, in most studies, isolates of C. difficile have generally been found to be susceptible to these (Drummond et al., 2003; Aspevall et al., 2006). However, a few studies have reported strains resistant to metronidazole or with reduced susceptibility to vancomycin (Brazier et al.,

Table 2. Range of MIC values and resistance rates from 116 isolates with the breakpoints used

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC range (µg ml⁻¹)</th>
<th>MIC₅₀ (µg ml⁻¹)</th>
<th>MIC₉₀ (µg ml⁻¹)</th>
<th>Breakpoint (µg ml⁻¹)</th>
<th>Resistance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vancomycin</td>
<td>1–4</td>
<td>2</td>
<td>4</td>
<td>≥8</td>
<td>0</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>≤0.5–4</td>
<td>1</td>
<td>2</td>
<td>≥8</td>
<td>0</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.5–≥32</td>
<td>≥32</td>
<td>≥32</td>
<td>≥8</td>
<td>94.8</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0.5–≥32</td>
<td>8</td>
<td>16</td>
<td>≥8</td>
<td>62.9</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>32–256</td>
<td>64</td>
<td>64</td>
<td>≥64</td>
<td>87.1</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>0.25–≥32</td>
<td>≥32</td>
<td>≥32</td>
<td>≥4</td>
<td>87.1</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>≤1–64</td>
<td>≤1</td>
<td>2</td>
<td>≥16</td>
<td>4.3</td>
</tr>
</tbody>
</table>
2001; Peláez et al., 2005). The first UK isolate of C. difficile with reduced susceptibility to metronidazole was reported in 2001 (Brazier et al., 2001).

Resistance to clindamycin was seen in 73 isolates (62.9%). Tetracycline resistance was low, with only five isolates with MICs \( \geq 16 \mu g \text{ ml}^{-1} \). MIC\(_{50}\) and MIC\(_{90}\) values for erythromycin were \( \geq 32 \mu g \text{ ml}^{-1} \), showing that the majority of the isolates were highly resistant to this antibiotic (\( n=110, 94.8\% \)). Similar high resistance rates to the antibiotics ceftriaxone and moxifloxacin (87.1\%) were also found. Their MIC\(_{50}\) values were high and the same as their MIC\(_{90}\) values.

Previously, moxifloxacin was reported to have good activity against Gram-positive bacilli including C. difficile (Hoogkamp-Korstanje & Roelofs-Willemsen, 2000), but reduced susceptibility to this antibiotic has been shown in several studies (Wilcox et al., 2000; Leroi et al., 2002). Clindamycin and ceftriaxone resistance rates did not show much difference from our previous study (Drummond et al., 2003).

**Antibiotic susceptibility in relation to molecular type**

Of the 116 isolates for which MICs were measured, 87 were of ribotype 001, 10 were of ribotype 106 and 19 were of other ribotypes. In terms of toxinotype, 112 were of toxinotype 0, with one isolate each of toxinotypes I, IV, V and XIII. The antibiotic resistance patterns of these 116 isolates are detailed in Table 3.

In a study from the UK, PCR ribotypes 001 and 106 were found to be more resistant to erythromycin (98 and 100\%, respectively) than other PCR ribotypes (John & Brazier, 2005). All of our ribotype 001 and 106 ribotypes were resistant to this antibiotic. We also found higher resistance levels to the antibiotics ceftriaxone and moxifloxacin, which were not tested in that study, among ribotype 001 and 106 isolates than among the other ribotypes. John & Brazier (2005) reported that clindamycin resistance was lower than erythromycin resistance in ribotypes 001 and 106, whilst ribotypes 015, 014, 005 and 002 had a higher frequency of resistance to clindamycin than to erythromycin. Of our isolates, only ribotype 002 had a higher clindamycin resistance level than erythromycin, but the frequency of clindamycin resistance was lower for ribotype 014, whilst both resistance rates were the same for ribotype 005. In a study in which clindamycin and fusidic acid resistances were determined (Aspevall et al., 2006), no particular relationship between PCR ribotypes and antibiotic resistance was found. The clindamycin resistance frequency in our study was lower than that found by Aspevall et al. (2006) (83\%). In a study from Australia, the MIC range for moxifloxacin (0.75 to \( \geq 32 \mu g \text{ ml}^{-1} \)) was found to be close to the resistance breakpoint with MIC\(_{50}\) and MIC\(_{90}\) values of 2 and 4 \( \mu g \text{ ml}^{-1} \), respectively (Leroi et al., 2002). Susceptibilities of clonal and distinct C. difficile strains from the UK to newer fluoroquinolones including moxifloxacin have been tested (Wilcox et al., 2000). Trovafloxacin and moxifloxacin were the most active fluoroquinolones with three- to fourfold more activity than other agents such as ciprofloxacin among genotypically distinct strains. Clonal strains that were epidemic ribotype 001 strains were sevenfold less susceptible to moxifloxacin compared with the distinct strains. The MIC range for this antibiotic was 0.12–16 \( \mu g \text{ ml}^{-1} \) and MIC\(_{50}\) and MIC\(_{90}\) values were 1 and 16 \( \mu g \text{ ml}^{-1} \), respectively (Wilcox et al., 2000). We found higher MIC\(_{50}\) and MIC\(_{90}\) values for moxifloxacin in our study. Most of the ribotype 001 (98.9\%, \( n=86 \)) and ribotype 106 (90\%, \( n=9 \)) isolates were resistant to moxifloxacin, whereas only six isolates (31.5\%) from other ribotypes were resistant in our study.

Only two isolates of flagellin gene restriction pattern I were observed. All of these restriction type I isolates were sensitive to tetracycline and moxifloxacin and resistant to erythromycin. One was resistant to both clindamycin and

**Table 3. Percentage antibiotic resistance rates by ribotype and toxinotype**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Percentage of isolates resistant to antibiotics based on:</th>
<th>Ribotype</th>
<th>Toxinotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin</td>
<td></td>
<td>001 (n=87)</td>
<td>106 (n=10)</td>
</tr>
<tr>
<td>Clindamycin</td>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td></td>
<td>62.1</td>
<td>70</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td></td>
<td>95.4</td>
<td>100</td>
</tr>
<tr>
<td>Tetracycline</td>
<td></td>
<td>98.9</td>
<td>90</td>
</tr>
<tr>
<td>Metronidazole</td>
<td></td>
<td>1.2</td>
<td>0</td>
</tr>
<tr>
<td>Vancomycin</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*As toxinotypes I, IV, V and XIII are represented by single isolates, the designations R (resistant) and S (sensitive) have been used rather than percentages.
ceftiraxone, whilst the other was sensitive to both antibiotics.

One of the two isolates carrying the binary toxin genes was resistant to erythromycin, clindamycin and tetracycline, whilst the other one was only resistant to ceftiraxone. Both isolates were sensitive to moxifloxacin.

Fifty-six of the 62 isolates that were typed by S-layer were tested for antibiotic susceptibility. Most of the S-type 4939 isolates (38/39) and all of the S-type 4741, 4938 and 4639 isolates (n=10, n=3 and n=2, respectively) were resistant to erythromycin. Most of the S-type 4939 and 4741 isolates were resistant to clindamycin, ceftiraxone and moxifloxacin. One isolate that belonged to S-type 4837 was sensitive to all antibiotics. This isolate was of ribotype 070, toxinotype 0 and fliC restriction type VII.

Multi-resistant strains

Seventy-two isolates were resistant to both erythromycin and clindamycin in our study, with resistance to both antibiotics being 62, 70 and 52.6 % in ribotypes 001, 106 and the others, respectively. Of toxinotype 0 and other toxinotype isolates, 61.6 and 75 % were resistant to both antibiotics, respectively. Only one fliC restriction pattern I isolate and one binary toxin gene-positive isolate resistant to these antibiotics were encountered. Macrolide–lincosamide–streptogramin B resistance in C. difficile is mostly encoded by the _ermB_ resistance determinant. This gene encodes a 23S rRNA methyltransferase that modifies the target site for the antibiotic and is a mobilizable, conjugative transposon, Tn5398 (Farrow _et al._, 2001). In recent years, some _ermB_-negative isolates with erythromycin and clindamycin resistance have been reported (Ackermann _et al._, 2003; Spigaglia & Mastrantonio, 2004; Pituch _et al._, 2006). Spigaglia & Mastrantonio (2004) could not find any _erm_ genes of other classes such as _ermA_, _ermC_, _ermF_, _ermQ_ and _mefA_ among these isolates. It has been suggested that resistance in _ermB_-negative resistant strains could be due to mutations within the target sequences in the 23S rRNA or efflux mechanisms or a new mechanism of resistance (Ackermann _et al._, 2003; Spigaglia & Mastrantonio, 2004; Pituch _et al._, 2006). We did not test our isolates for resistance genotypically.

A total of 66 isolates were resistant to the three antibiotics erythromycin, clindamycin and ceftiraxone and 96 isolates were resistant to erythromycin, ceftiraxone and moxifloxacin. Ribotypes 001 and 106 had higher resistances (95.4 and 90 %, respectively) to the antibiotics erythromycin, ceftiraxone and moxifloxacin when compared with other PCR ribotype groups (21 %). Ackermann _et al._ (2001) suggested that resistance to moxifloxacin might be due to amino acid substitution in the DNA gyrase. They also found that moxifloxacin-resistant strains that were selected _in vitro_ had wild-type _gyrA_ sequences.

In our study, only one isolate was resistant to five antibiotics: erythromycin, clindamycin, moxifloxacin, ceftiraxone and tetracycline; it was of ribotype 001 and toxinotype 0. Ackermann _et al._ (2003) reported resistances to the antibiotics erythromycin, clindamycin and moxifloxacin as 27, 36 and 12 %, respectively, among 192 isolates tested. They found that moxifloxacin resistance was almost always detected together with resistance to erythromycin and clindamycin (12.5 %). In our study, 110 erythromycin-resistant isolates were found of which 100 (90.9 %) were resistant to moxifloxacin and only one out of six erythromycin-sensitive isolates was resistant to moxifloxacin. Among the 73 clindamycin-resistant isolates, 64 (87.7 %) were also resistant to moxifloxacin. Of the 72 isolates resistant to both erythromycin and clindamycin, 64 (88.9 %) were resistant to moxifloxacin. Additionally, we found that all but one of the 64 isolates that were resistant to these three antibiotics were also resistant to ceftiraxone and these multi-resistant isolates were mostly of ribotype 001 (n=53, 84.1 %).

As the 027 type has a characteristic antibiotic resistance pattern – resistant to erythromycin, susceptible to clindamycin and resistant to moxifloxacin (Kuijper _et al._, 2006b) – this could be part of an algorithm to identify 027 strains. However, in our study we identified 36 strains that were resistant to erythromycin, susceptible to clindamycin and resistant to moxifloxacin. Thirty-two of these strains were of ribotype 001, three were of ribotype 106 and one was of 014, and all of them were of toxinotype 0. This questions the usefulness of this approach to detect 027 strains.

We are aware that a Europe-wide surveillance study has been performed (F. Barbut and others, unpublished) and some strains for this study were collected in Scotland. However, there was no overlap in strains between these studies as those for the European surveillance study were collected earlier in 2005.

The results obtained from this study demonstrate clearly the complexity of the strains of _C. difficile_ in our area. If characterized purely on ribotyping, it would appear that most of the strains are closely related. However, the use of other typing methods, especially antibiotic resistance patterns, demonstrates wide variation among strains.

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REFERENCES


