Emergence of reduced susceptibility to metronidazole in *Clostridium difficile*

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**Objectives:** Antimicrobial treatment for *Clostridium difficile* infection (CDI) has typically been metronidazole, although reports have questioned the efficacy of this option. We screened recently isolated *C. difficile* (2005–06) for susceptibility to metronidazole and compared results for historic isolates (1995–01).

**Methods:** *C. difficile* ribotypes 001 (n = 86), 106 (n = 81) and 027 (n = 48) and isolates from the 10 other most prevalent ribotypes in Leeds (n = 57) were screened using spiral gradient endpoint analysis (SGE). *C. difficile* with metronidazole SGE MICs >6 mg/L were analysed further by agar incorporation and Etest. Multiple-locus variable-number tandem-repeat analysis (MLVA) typing was performed for 28 *C. difficile* isolates.

**Results:** No reduced metronidazole susceptibility was observed in *C. difficile* ribotypes 106 and 027 (geometric mean SGE MICs 1.11 and 0.90 mg/L, respectively). In contrast, 21 (24.4%) *C. difficile* ribotype 001 demonstrated reduced susceptibility to metronidazole (geometric mean SGE MICs 3.51 mg/L, P < 0.001). Variations in susceptibility were observed relating to the method and media, but increased metronidazole MICs were confirmed by an agar incorporation method. Geometric mean agar incorporation MICs for historic *C. difficile* ribotype 001 (n = 72) were 1.03 (range 0.25–2) mg/L compared with 5.94 (4–8) mg/L (P < 0.001) for recent isolates displaying reduced metronidazole susceptibility. MLVA typing revealed two clonal complexes of *C. difficile* with reduced susceptibility to metronidazole.

**Conclusions:** We have demonstrated the emergence of reduced susceptibility to metronidazole in 24.4% of the recent *C. difficile* ribotype 001 isolates from our institution. Our observations could have implications in the clinical setting due to the poor penetration of metronidazole into the colon.

Keywords: antibiotics, diarrhoea, pseudomembranous colitis

**Introduction**

Treatment options for *Clostridium difficile* infection (CDI) have changed little over the past two decades, and the great majority of cases are prescribed either oral metronidazole or oral vancomycin.¹ Early studies demonstrated little difference between metronidazole and vancomycin in terms of response or recurrence rates,²,³ although time to response was shorter with the latter.⁴ More recent reports have questioned the efficacy of metronidazole therapy for CDI, particularly in severe cases and those attributable to an apparently hypervirulent *C. difficile* PCR ribotype 027 (NAP1/BI).⁵–⁷ One recent study reported that vancomycin was superior to metronidazole for the treatment of severe CDI and similarly effective in treating mild/moderate CDI.⁸ There have been only occasional reports of resistance to metronidazole (CLSI resistance breakpoint MIC ≥ 32 mg/L) and vancomycin (CLSI resistance breakpoint MIC ≥ 16 mg/L).⁹,¹⁰ Indeed, we evaluated the susceptibilities of epidemic (PCR ribotype 001) and genotypically distinct (by PCR ribotyping) *C. difficile* isolates from 1995/96 and 2000/01 and failed to
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demonstrate any isolates with reduced susceptibility to either metronidazole or vancomycin. Other studies have demonstrated small numbers of C. difficile with reduced susceptibility to metronidazole, although sample sizes in these studies have been relatively small. The largest evaluation of metronidazole susceptibility in C. difficile was performed by the Anaerobe Reference Laboratory (Cardiff, UK), in which only one of >1000 isolates demonstrated reduced susceptibility (MIC 16 mg/L).

Continued surveillance for resistance to therapeutic antimicrobials in C. difficile is essential, given the limited array of antimicrobials available to treat CDI. Therefore, we screened a large number of isolates from the most prevalent C. difficile PCR ribotypes (001, 106 and 027) during 2005–06 and also a group that comprised the 10 other most commonly encountered PCR ribotypes isolated in Leeds, UK, for reduced susceptibility to metronidazole or vancomycin. We used multiple methods to compare the MICs for isolates that displayed reduced susceptibility to metronidazole, compared the results with historical strains and investigated the relatedness of isolates with altered phenotypes using highly discriminatory DNA fingerprinting.

Materials and methods

C. difficile strains

In 2005, a prospective surveillance programme was commenced in Leeds, which aimed to culture and fingerprint C. difficile (by PCR ribotyping) from every cytotoxin-positive faecal sample detected by the routine diagnostic microbiology laboratory. Between 2005 and 2006, 1134 C. difficile were isolated and PCR ribotyped. Following PCR ribotyping, spore preparations of all C. difficile isolates were maintained at −70°C. During this period, the most commonly encountered C. difficile PCR ribotypes were 001 (33%), 106 (34%) and 027 (4%). In the present study, every third isolate chronologically of C. difficile PCR ribotypes 001 (n = 86) and 106 (n = 81) was examined, in addition to all C. difficile PCR ribotype 027 (n = 48) and a selection of the other 10 most commonly isolated PCR ribotypes (n = 57). The latter group consisted of C. difficile PCR ribotypes: 002 (n = 6), 005 (n = 6), 014/20 (n = 6), 015 (n = 5), 023 (n = 7), 049 (n = 6), 078 (n = 4) and also unknown ribotypes LU6 (n = 6), LU8 (n = 5) and LU14 (n = 5). Unknown ribotypes refer to C. difficile strains that did not match any confirmed PCR ribotype in the Leeds library. Ribotyping classifications were based on the nomenclature of the Anaerobe Reference Laboratory. C. difficile included in this study were single patient isolates; repeat isolates from the same patient were excluded.

Screening for evidence of reduced metronidazole susceptibility

C. difficile were cultured from freezer vials by inoculating onto Brazer’s CCEY agar (Bioconnections, UK) supplemented with 5 mg/L lysozyme, 2% lysed horse blood (E&O Laboratories, UK) and cycloserine/cephalexin (Bioconnections), with incubation at 37°C for 48 h in an anaerobic cabinet (Don Whitley Scientific, UK). Spiral gradient endpoint analysis (SGE) was used to screen C. difficile isolates for reduced susceptibility to metronidazole. Briefly, stock solutions of 1000 mg/L metronidazole (Sigma-Aldrich, UK) were prepared in de-ionized water and sterilized by filtration through 0.22 μm syringe filters. Brazer’s CCEY agar plates (without cycloserine/cefotaxin and egg yolk) were dried for 10 min at 37°C, and a logarithmic spiral gradient of metronidazole was applied onto the surface using a spiral plater (WASP, Don Whitley Scientific). Metronidazole gradient agar was left at room temperature for 1 h in order to allow the agar to absorb the metronidazole. C. difficile and controls were cultured anaerobically (37°C) overnight in Schaedler’s anaerobic broth (Oxoid, UK). Bacteroides fragilis ATCC 25285 was used as a metronidazole-susceptible control and C. difficile PCR ribotype 010 (metronidazole MIC 8–16 mg/L) as a metronidazole-reduced susceptibility control. Test C. difficile isolates (~107 cfu/mL) and one of each control strain were inoculated in duplicate onto the agar surface using a sterile cotton swab. D values (mm) were measured and converted to MICs using the method of Paton et al. Any C. difficile with metronidazole MICs ≥ 6 mg/L were subjected to further testing.

MIC determination using agar incorporation

C. difficile isolates identified with reduced susceptibility to metronidazole by SGE had MICs re-measured by agar incorporation using the methods of Freeman et al.11 and the CLSI.19 Additionally, in order to assess the effect of agar base formulation on metronidazole and vancomycin MICs, C. difficile and control organisms raised in Schaedler’s anaerobic broth were inoculated onto Brucella agar (Oxoid) incorporating haemin (5 mg/L), vitamin K1 (1 mg/L) and 5% horse blood (E&O Laboratories), and MICs were determined. B. fragilis ATCC 25285, Staphylococcus aureus ATCC 29213 and Enterococcus faecalis ATCC 29212 were used as control organisms. All MICs were performed in duplicate. MIC endpoints were identified as the lowest concentration where a marked reduction in growth was observed compared with the growth control.

MIC determination using Etest

Metronidazole and vancomycin MICs were determined using Etest (AB Biodisk, Sweden) on both Wilkins Chalgren and Brucella agar bases. C. difficile (~107 cfu/mL) were cultured anaerobically overnight (37°C) in Schaedler’s anaerobic broth and inoculated onto the surface of Wilkins Chalgren agar and Brucella agar using a sterile cotton swab. Inoculated agars were allowed to dry, following which a single Etest strip was placed onto each agar surface. Agar plates were incubated anaerobically (37°C) for 24 h, and MICs were determined following the manufacturer’s instructions.

Comparison of MICs obtained for historical isolates

MICs of metronidazole for historic C. difficile ribotype 001 from 1995 to 2001 (n = 72)11 were determined alongside recent C. difficile PCR ribotype 001 that was identified as having reduced susceptibility to metronidazole. The method used for this MIC comparison was agar incorporation.

Multiple-locus variable-number tandem-repeat analysis (MLVA) typing

The genetic relatedness of C. difficile that displayed reduced susceptibility to metronidazole (n = 22) was analysed alongside fully susceptible isolates of the same PCR ribotype (n = 6) using MLVA. MLVA was performed using the previously described method with one alteration: a new reverse primer was developed for the marker CaG8: 5’ ACCAAAAATTCTAACCACAC 3’. The genetic relationships among the genotypes were determined by clustering them according to the MLVA type using the number of differing
loci and the summed absolute distance as coefficients for calculating the minimum-spanning tree, as described by Marsh et al.\textsuperscript{21} using the BioNumerics program (version 4.6, Applied Maths, Belgium). Briefly, the summed absolute distance between two MLVA-typed isolates is the summed tandem-repeat difference (STRD) at all seven variable number of tandem-repeat loci. Isolates with an STRD ≤2 were defined as genetically related. Clonal complexes were defined by an STRD ≤2, provided that the isolates were single-locus variants or double-locus variants of each other.\textsuperscript{21} This analysis was carried out with the workers blinded to the phenotypes of the 28 \textit{C. difficile} isolates.

### Statistical analysis

Metronidazole MICs for \textit{C. difficile} PCR ribotypes 001, 106, 027 and the other 10 most commonly encountered PCR ribotype group were tested for normality and homogeneity of variance. Median MICs for these groups were analysed using a Mann–Whitney test. Log$_2$ (MICs) for historical \textit{C. difficile} and recent \textit{C. difficile} strains identified with reduced susceptibility to metronidazole by agar incorporation were tested for normality and homogeneity of variance and analysed for statistical significance using a Mann–Whitney test. All statistical analyses were performed using Minitab version 14 and SPSS version 14.0, and \textit{P} < 0.05 was considered statistically significant.

### Results

#### Metronidazole MICs by SGE

Geometric mean metronidazole MICs for PCR ribotypes 001, 106, 027 and the mixed ribotype group were 3.51, 1.11, 0.90 and 0.32 mg/L, respectively (Table 1). Median MICs for PCR ribotype 001 were statistically significantly higher than PCR ribotypes 106 and 027 (median MICs 4.24 versus 1.03 and 0.77 mg/L, respectively; \textit{P} < 0.001 for both ribotype groups). Also, the median MIC for the mixed ribotype group was significantly lower than that for any of the three epidemic ribotype groups examined (0.21 mg/L; \textit{P} < 0.001 for all three ribotype groups). Twenty-nine \textit{C. difficile} isolates were identified with MICs ≥6 mg/L; 95% (21/22) were PCR ribotype 001, with the remaining strain identified as PCR ribotype LU6. Metronidazole MICs for \textit{B. fragilis} ATCC 25285 and \textit{C. difficile} PCR ribotype 010\textsuperscript{15} controls were within expected limits (0.25 and 15.9 mg/L, respectively).

#### MICs by Etest

Geometric mean metronidazole MICs were 3.12 mg/L (range 1–8 mg/L) on Wilkins Chalgren agar and 3.37 mg/L (range 2–6 mg/L) on Brucella agar base. Corresponding vancomycin geometric mean MICs were 2.14 and 2.28 mg/L, respectively. One \textit{C. difficile} isolate demonstrated a vancomycin MIC of 8 mg/L on both Wilkins Chalgren and Brucella agars. Geometric mean MICs by the CLSI guidelines\textsuperscript{10} were determined at two geographically distinct laboratories and were lower than those observed by the method of Freeman et al.\textsuperscript{11} Historic \textit{C. difficile} PCR ribotype 001 metronidazole geometric mean MICs determined (following the method of Freeman et al.\textsuperscript{11}) alongside those for recent \textit{C. difficile} PCR ribotype 001 displaying reduced susceptibility to metronidazole were 1.03 (range 0.25–2) and 5.94 mg/L (range 4–8), respectively (\textit{P} < 0.001).

#### MLVA typing

Highly discriminatory DNA fingerprinting using MLVA of 22 reduced susceptibility isolates and 6 fully susceptible isolates revealed that 24 of these isolates were genetically related (STRD ≤10). Two clonal complexes (CC-I and CC-2, i.e. STRD ≤2) of \textit{C. difficile} PCR ribotype 001 were recognized (Figure 1). Of the 22 reduced susceptibility strains, 21 were genetically related and 15 belonged to one of the two clonal complexes. Clonal complexes were not recognized within the six susceptible strains.

### Discussion

CDI is a disease of increasing importance with worldwide epidemics. These are attributable to specific \textit{C. difficile} PCR

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**Table 1. SGE metronidazole MICs (mg/L) for \textit{C. difficile} isolated at the Leeds General Infirmary in 2005–06**

<table>
<thead>
<tr>
<th>MIC</th>
<th>Ribotype 001 (n = 86)</th>
<th>Ribotype 106 (n = 81)</th>
<th>Ribotype 027 (n = 48)</th>
<th>10 most prevalent other ribotypes (n = 57)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC$_{50}$</td>
<td>4.16</td>
<td>1.03</td>
<td>0.80</td>
<td>0.21</td>
</tr>
<tr>
<td>MIC$_{90}$</td>
<td>15.89</td>
<td>1.82</td>
<td>1.40</td>
<td>2.09</td>
</tr>
<tr>
<td>Range</td>
<td>0.27–15.90</td>
<td>0.36–2.57</td>
<td>&lt;0.15–4</td>
<td>&lt;0.15–14.50</td>
</tr>
<tr>
<td>Geometric mean</td>
<td>3.51*</td>
<td>1.11</td>
<td>0.90</td>
<td>0.32</td>
</tr>
</tbody>
</table>

*\textit{P} < 0.001.
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Table 2. Agar incorporation metronidazole and vancomycin MICs for C. difficile identified with reduced susceptibility (n = 22) to metronidazole by SGE

<table>
<thead>
<tr>
<th>Antimicrobial/MIC (mg/L)</th>
<th>Wilkens Chalgren</th>
<th>Brucella</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metronidazole</td>
<td></td>
<td></td>
</tr>
<tr>
<td>geometric mean</td>
<td>9.19</td>
<td>4.83</td>
</tr>
<tr>
<td>range</td>
<td>8–16</td>
<td>4–8</td>
</tr>
<tr>
<td>Vancomycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>geometric mean</td>
<td>2.14</td>
<td>2.28</td>
</tr>
<tr>
<td>range</td>
<td>1–8</td>
<td>2–8</td>
</tr>
</tbody>
</table>

Inocula were raised in Schaedler’s anaerobic broth.

Table 3. Etest metronidazole and vancomycin MICs for C. difficile (n = 22) identified with reduced susceptibility to metronidazole by SGE

<table>
<thead>
<tr>
<th>Antimicrobial/MIC (mg/L)</th>
<th>Wilkens Chalgren</th>
<th>Brucella</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metronidazole</td>
<td></td>
<td></td>
</tr>
<tr>
<td>geometric mean</td>
<td>3.12</td>
<td>3.37</td>
</tr>
<tr>
<td>range</td>
<td>1–8</td>
<td>2–6</td>
</tr>
<tr>
<td>Vancomycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>geometric mean</td>
<td>1.47</td>
<td>1.53</td>
</tr>
<tr>
<td>range</td>
<td>1–3</td>
<td>0.75–3</td>
</tr>
</tbody>
</table>

ribotypes, including PCR ribotype 001 and an apparently hyper-virulent strain, PCR ribotype 027, also known as NAP1 (by pulsed-field gel electrophoresis) and BI (by restriction endonuclease analysis). It is noteworthy that recent epidemics, primarily caused by C. difficile ribotype 027, have been associated with poor efficacy of antibiotic therapy. To date, poor metronidazole efficacy in treating CDI does not appear to be associated with reduced susceptibility of the organism.

Few laboratories are carrying out surveillance for changes in antibiotic susceptibility in C. difficile, principally because routine culture of the bacterium is uncommon. In the present study, we screened the most prevalent C. difficile PCR ribotypes (106, 001 and 027) and also a group comprising the 10 most commonly isolated other PCR ribotypes from 2005 to 2006 in Leeds for susceptibility to metronidazole. We demonstrated reduced susceptibility to metronidazole in 24.4% of the C. difficile PCR ribotype 001 isolates, but not in the other most prevalent PCR ribotypes (106 and 027). Using historical controls, we have shown that this is a relatively new phenomenon, as reduced susceptibility to metronidazole was not present in isolates recovered as recently as 2001. We found evidence using highly discriminatory DNA fingerprinting that supports clonal spread of isolates with reduced susceptibility to metronidazole. Indeed, of 22 strains with reduced susceptibility, 15 belonged to one of two clonal complexes. As systematic antimicrobial susceptibility testing of C. difficile is not carried out at our institution, it is possible that this phenotype may have been present earlier but was undetected. The development of antimicrobial resistance usually requires antibiotic exposure followed by the selection and expansion of clones. It might be expected, therefore, that the development of metronidazole reduced susceptibility is more likely to be seen in prevalent as opposed to uncommon C. difficile PCR ribotypes within an institution. It will be important to monitor prospectively for the emergence of reduced susceptibility to metronidazole or vancomycin, particularly in prevalent C. difficile PCR ribotypes.

The clinical significance of the observed shift in the MICs of metronidazole is unclear. Review of medical records of patients from whom reduced metronidazole-susceptibility C. difficile were recovered could be helpful. However, suboptimal response and/or recurrence rates may be difficult to distinguish from expected results, and the difficulty of defining clinical success must be noted in a predominantly frail elderly population with frequent co-morbidities and polypharmacy. A recent prospective observational study examined the clinical and microbiological response to treatment of CDI with 10 days of metronidazole or vancomycin. Vancomycin-treated patients had a better microbiological response between day 1 and 5 of therapy, as measured by the eradication of C. difficile and the resolution of diarrhea. Unfortunately, neither the faecal concentrations of metronidazole nor the in vitro antimicrobial susceptibilities of C. difficile isolates were examined. In theory, given the pharmacokinetic profile of metronidazole in humans, only a small elevation in metronidazole MIC could have a marked effect on treatment efficacy in CDI. Mean antibiotic concentrations reported in faeces of patients receiving oral metronidazole ranged from <0.25 to 9.5 mg/L, and drug concentration decreased as diarrhea resolved. Thus, C. difficile strains with metronidazole MICs of 8–16 mg/L may not be inhibited by in vivo antibiotic concentrations in faeces. Conversely, the single C. difficile PCR ribotype 001 isolate that we found with a vancomycin MIC of 8 mg/L, when measured on both Brucella and Wilkins Chalgren agars, is unlikely to be clinically significant as faecal vancomycin concentrations after an oral antibiotic course range from 520 to 2200 mg/L.

C. difficile PCR ribotype 001 was by far the most commonly isolated ribotype in the UK (~55% of the isolations in 1995–2003). In our institution, C. difficile PCR ribotype 001 was even more prevalent with ~80% of the C. difficile isolates belonging to this ribotype in 2000–01. Marked changes in the UK distribution of C. difficile PCR ribotypes have occurred since 2003. For example, the most common PCR ribotypes in 2007 detected by the C. difficile Ribotyping Network for England were 027 (48%), 106 (13%) and 001 (10%). Such shifts in C. difficile ribotype prevalence again emphasize the need for prospective surveillance to identify altered susceptibility to metronidazole or vancomycin.

We found that experimental methodology clearly affected the magnitude of measured metronidazole MICs for C. difficile. Despite the consistent demonstration of increased metronidazole MICs in C. difficile PCR ribotype 001 by both spiral gradient endpoint and agar incorporation methods, the results obtained by Etest and CLSI agar incorporation methods failed to correlate. Agar base composition and broth inoculum may affect C. difficile metronidazole MICs, and discrepancies between agar incorporation and Etest metronidazole MICs have been reported previously. We stress, however, that the comparison of metronidazole
susceptibilities of recent versus historical C. difficile PCR ribotype 001 isolates from our institution demonstrated statistically significantly different MICs. Crucially, these differences in MICs were demonstrated using an agar incorporation method that was identical to that employed previously,\textsuperscript{11,29,33} with a collection of strains with known metronidazole susceptibilities.\textsuperscript{11} Wilkins Chalgren agar was formerly the reference medium recommended by the CLSI (formerly NCCLS) for susceptibility testing of anaerobes. Experience in our laboratory has suggested that more reproducible growth of C. difficile occurs on Wilkins Chalgren in comparison with other agar bases, including supplemented Brucella agar.

Paton et al.\textsuperscript{18} validated SGE testing and reported good correlation (correlation coefficients 0.74–0.97 for eight antibiotics) using agar incorporation and broth dilution methods. Preliminary investigations in our laboratory indicated comparable metronidazole MICs using agar incorporation and SGE methods (data not shown). We found that SGE MICs were significantly lower for C. difficile in the group composed of the 10 other most prevalent PCR ribotypes than for PCR ribotypes 001, 106 and 027 \((P < 0.001)\). Burns et al.\textsuperscript{34} recently reported that there were no significant differences in metronidazole MICs between three epidemic C. difficile ribotypes (001, 106 and 027). Although all the epidemic strains were significantly less susceptible to metronidazole than non-epidemic strains (1.16–1.5 versus 0.37 mg/L),\textsuperscript{34} the MICs were all \(\leq 1.5\) mg/L.

Metronidazole resistance studies in Helicobacter pylori and B. fragilis identified a family of nitroimidazole genes (nimA–E) associated with resistance. Homologous genes have been identified in Clostridium tetani and Clostridium bifermentans.\textsuperscript{35,36} We did not detect nim genes in a prior study that identified the first metronidazole-resistant C. difficile strain in the UK;\textsuperscript{15} the strain used as a positive control in the present study. The mechanism(s) of reduced susceptibility to metronidazole in C. difficile PCR ribotype 001 from our institution are yet to be elucidated. Reduced entry of metronidazole or enhanced efflux of the drug may explain our observations, but further studies are required to delineate mechanisms of reduced susceptibility.

In conclusion, we have demonstrated the emergence of reduced susceptibility to metronidazole in approximately a quarter of recent C. difficile PCR ribotype 001 isolates, but not in other epidemic C. difficile. Changes in susceptibility may be missed by some MIC methods. Given the pharmacokinetic profile of metronidazole in humans, the magnitude of reduction

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Minimum spanning tree representation of MLVA data for C. difficile. White circles indicate metronidazole-susceptible isolates; grey circles indicate C. difficile with reduced susceptibility to metronidazole. Two circles contain more than one isolate: these are 100% homologous at all seven VNTR loci. All C. difficile were PCR ribotype 001 except number 28 (LU6). The numbers between the circles represent the summed tandem-repeat differences (STRDs) between MLVA types. Thick lines represent single-locus variants, double lines represent double-locus variants and the dotted and dashed lines represent triple- and pentuple-locus variants. CC-1 and CC-2 represent clonal complexes with an STRD \(\leq 2\).}
\end{figure}
in metronidazole susceptibility may have clinical implications, but further studies are required to assess this possibility. Continued surveillance of susceptibility to the two main antibiotics used to treat CDI, especially in prevalent C. difficile clones, is essential to detect the emergence of resistance.

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