Multidrug resistance in European *Clostridium difficile* clinical isolates

Patrizia Spigaglia, Fabrizio Barbanti and Paola Mastrantonio* on behalf of the European Study Group on *Clostridium difficile* (ESGCD)†

Department of Infectious, Parasitic and Immune-mediated Diseases, Istituto Superiore di Sanità, Rome, Italy

*Corresponding author. Tel: +39-06-49902335; Fax: +39-06-49902886; E-mail: paola.mastrantonio@iss.it

†Members of the European Study Group on *Clostridium difficile* (ESGCD) are listed in the Acknowledgements section.

Received 25 March 2011; returned 6 May 2011; revised 17 June 2011; accepted 20 June 2011

Objectives: Multidrug resistance and antibiotic resistance mechanisms were investigated in 316 *Clostridium difficile* clinical isolates collected during the first European surveillance on *C. difficile* in 2005.

Methods: MICs of eight different antibiotics were determined using Etest. Reserpine- and carbonyl cyanide m-chlorophenylhydrazone-sensitive efflux was tested using the agar dilution method. Molecular analysis of the resistance mechanisms was performed using PCR assays, PCR mapping and sequencing.

Results: One hundred and forty-eight *C. difficile* strains were resistant to at least one antibiotic and 82 (55%) were multidrug resistant. In particular, 48% of these isolates were resistant to erythromycin, clindamycin, moxifloxacin and rifampicin. New genetic elements or determinants conferring resistance to erythromycin/clindamycin or tetracycline were identified. Even if most multiresistant strains carried an *erm*(B) gene, quite a few were *erm*(B) negative. In-depth analysis of the underlying mechanism in these isolates was carried out, including analysis of 23S rDNA and the ribosomal proteins L4 and L22. Interestingly, resistance to rifampicin was observed in multidrug-resistant strains in association with resistance to fluoroquinolones. Mutations in the rpo(B) and gyrA genes were identified as the cause of resistance to these antibiotics, respectively.

Conclusions: Characterization of multidrug-resistant *C. difficile* clinical isolates shows that antibiotic resistance is changing, involving new determinants and mechanisms and providing this pathogen with potential advantages over the co-resident gut flora. The present paper provides, for the first time, a comprehensive picture of the different characteristics of multidrug-resistant *C. difficile* strains in Europe in 2005 and represents an important source of data for future comparative European studies.

Keywords: antibiotics, resistance mechanisms, surveillance

Introduction

*Clostridium difficile* is the leading cause of hospital-acquired diarrhoea, ranging from mild cases to severe pseudo-membranous colitis, collectively known as *C. difficile* infection (CDI). Antimicrobial therapy plays a central role in the development of CDI, disrupting the normal intestinal flora and creating conditions that favour acquisition and proliferation of *C. difficile*.1,2

Some local studies indicate that the percentage of *C. difficile* multidrug-resistant strains is variable and ranges between 2.5% and 66%,3–7 but no data on a large number of isolates collected throughout several European countries during a surveillance study are available.

Multidrug resistance may be generated by different mechanisms, such as accumulation of genes coding for resistance to different antibiotics or genomic mutations altering antibiotic target sites. In *C. difficile*, conjugative transposons are involved in resistance to the macrolide/lincosamide/streptogramin B (MLS$_B$) group of antibiotics, to tetracycline and to chloramphenicol. Historically, most of the prevalent *C. difficile* types in humans were resistant to clindamycin and/or erythromycin, two antibiotics belonging to the MLS$_B$ group.8–10 A specific mobilizable conjugative transposon called Tn5398 has been identified in some *C. difficile* strains, including *C. difficile* 630,11 but other isolates carried different elements.12 Nevertheless, an increasing number of resistant *erm*(B)-negative *C. difficile* isolates have been described, including the epidemic strain *C. difficile* B1/NAP1/027.3,7,11–15

Resistance to tetracycline and chloramphenicol has also been observed in both historic and recent isolates. In *C. difficile*, tetracycline resistance is commonly conferred by a tet(M) gene carried by a Tn5397 transposon,16 but some strains can harbour elements belonging to the Tn916 family, which is widely dispersed in both Gram-positive and -negative bacteria.17
Another family of transposons, the Tn4451/Tn4453 family,17 is responsible for resistance to chloramphenicol in this pathogen. These elements carry a cat(D) gene, which encodes for a chloramphenical acetyltransferase (CAT) inactivating the antibiotic.

Genomic mutations are involved in resistance to both fluoroquinolones and rifampicin, the latter recently proposed to treat ribonil acetyltransferase (CAT) inactivating the antibiotic.

One hypervirulent (MIC creep) and heteroresistance have been observed.27,28 Only one C. difficile clinical isolates, including the hypervirulent C. difficile BI/NAP1/027, show the substitution Thr-82 → Ile in GyrA, conferring resistance to fluoroquinolones, in particular to moxifloxacin,21,22 whereas resistance to rifampicin has been associated with amino acid substitutions between position 488 and 548 of the β subunit of RNA polymerase RpoB.23,24

The current standard treatment for CDI is metronidazole or vancomycin, with vancomycin as the antibiotic of choice for severe CDI. Only a few C. difficile strains resistant to metronidazol have been described in the world.25,26 but increases in MIC (MIC creep) and heteroresistance have been observed.27,28 Only one C. difficile strain resistant to vancomycin has been described, and decreased susceptibility to this antibiotic has been reported in some studies.26,29 The mechanisms of reduced susceptibility to metronidazole and vancomycin in this bacterium are still unknown.

In this study, 316 C. difficile clinical isolates, collected in 2005 during the first European prospective survey of C. difficile infections,30 were analysed for their susceptibility patterns and for the mechanisms of resistance to eight different classes of antibiotics (clindamycin, chloramphenicol, erythromycin, moxifloxacin, metronidazole, rifampicin, tetracycline and vancomycin), with a particular interest to multidrug-resistant strains.

The present paper gives a comprehensive picture of antibiotic resistance in European C. difficile clinical isolates and represents a precious source of data for future comparative surveillance studies.

Materials and methods

C. difficile isolates

The 316 C. difficile clinical isolates analysed in this study were collected during a prospective study conducted from April to June 2005 in 38 different hospitals in 14 European countries (Belgium, France, Germany, Great Britain, Greece, Hungary, Ireland, Italy, Poland, Spain, Sweden, Switzerland, The Netherlands and Turkey).30 All strains were isolated from symptomatic patients. Diarrhoea was defined as two or more loose stools per day for at least 2 days. A case of CDI was defined as a diarrhoea occurring in a patient without history of hospitalization during the previous month. Otherwise, CDI was defined as healthcare-associated.

All strains were typed during that study by PCR ribotyping using the method described by Stubbs et al.31

Antibiotic susceptibility

During the study performed in 2005, MICs of erythromycin, clindamycin, moxifloxacin, tetracycline, vancomycin and metronidazole were determined by Etest (AB Biodisk, Solna, Sweden) on brucella agar (BA) plates containing vitamin K1 (0.5 μg/mL), haemin (5 μg/mL) and 5% defibrinated sheep red blood cells. Quality control strains used for susceptibility testing included Bacteroides thetaiotaomicron ATCC 29741. In this study we used the same method to evaluate MIC values of rifampicin, chloramphenicol, levofloxacin, gatifloxacin and ciprofloxacin. The susceptibility test to the last three antibiotics was evaluated only on those isolates resistant to moxifloxacin.

In accordance with the guidelines established by the CLSI for anaerobic bacteria,22 the breakpoints used were 8 mg/L for erythromycin, clindamycin and moxifloxacin, 16 mg/L for tetracycline, and 32 mg/L for metronidazole and chloramphenicol. The breakpoints for rifampicin and vancomycin were 4 and 16 mg/L, respectively, in accordance with the CLSI interpretative categories approved for Staphylococcus aureus,33,34 since no values were provided for anaerobes.

Reserpine- and carbonyl cyanide m-chlorophenylhydrazine (CCCP) sensitive efflux was tested by agar dilution using plates supplemented with erythromycin or clindamycin in the presence or absence of these two potent inhibitors. Four sets of BA plates were made with doubling dilutions of the appropriate antibiotic. One set was used without inhibitors, whereas 20 or 80 mg/L reserpine (Sigma Chemical Co., St Louis, MO, USA) and/or 100 μM CCCP (Sigma Chemical Co.) were added to the other three sets of plates. C. difficile strains with a 4-fold or greater reduction in MIC in the presence of inhibitors were considered positive for reserpine- and CCCP-sensitive efflux.

Molecular analysis of mechanisms of resistance

Molecular analysis of resistance mechanisms was performed using PCR assays, PCR mapping and sequencing. Genomic DNA was used as template for PCR assays and was isolated using the NUCLEOBOND® buffer set III and NucleoBond® AXG 20 (Macherey-Nagel, Düren, Germany), whereas PCR fragments were used as template for sequencing after purification with the NucleoSpin extract kit (Macherey-Nagel). Sequence analysis was performed using BLAST software, ORF Finder and the European Bioinformatics Institute Clustal W server web site link (http://blast.ncbi.nlm.nih.gov/Blast.cgi, http://www.ncbi.nlm.nih.gov/orf2orf, html and http://www.ebi.ac.uk/Tools/msa/clustalw2/), respectively.

erm(B) genes were detected by the amplification of an internal fragment using the primer pair E5/E6,7 whereas erythromycin resistance determinants were characterized using a set of primers that amplify seven overlapping PCR products.12 We used the primer pairs described by Patterson et al.35 to detect the presence of other classes of erm (A, C, E, F and Q).

The primer pair TETd/TETMr was used to detect tet(M) in C. difficile strains resistant to tetracycline.36 The indX gene, a marker for Tn5397-like elements, and the int gene, a marker for Tn916-like elements, were detected as previously described.36 Other tet classes (A, AP, O, Q and W) were investigated using a specific set of primers already published.35 Characterization of tet(W) genes and their flanking regions was carried out by PCR amplification and sequencing by the primer couples used for the analysis of the tet(W) region in C. difficile CDS.37

A cat(D) gene internal fragment was amplified with primer CL1/CL2.7 The tnpX gene, a marker for the Tn4453 family of transposons was amplified using primers TnCLHf1 5'-AAGTAAACTCTTGACTGC-3' and TnCLHr1 5'-GATGCTGTTCTCCGTGTTGC-3', designed on the tnpX nucleotide sequence AF226276. The expected PCR product was of 0.6 kb and the PCR conditions used were 94°C for 5 min; 30 cycles of 30 s at 94°C, 30 s at 56°C and 30 s at 72°C; and a final extension of 5 min at 72°C.

Analysis of 23S genes was performed by PCR mapping and sequencing using six primer pairs already described.38 Each primer pair amplifies the target region of all the 23S genes present in the genome, which are more than one in C. difficile.39 so the derived chromatogram represents the analysis of a number of DNA fragments. For the analysis of ribosomal proteins L4 and L22, two specific primer pairs were designed on C. difficile 630: R20291 and CD196 genome sequences (GenBank accession numbers FNS45816 and FNS39870, respectively). The primer
pair L4-1f 5′-TACCCAGGCTAAAGGTCA-3′ and L4-4r 5′-GTTTTGGTGC CATCTTAG-3′ amplifies a DNA fragment of 840 bp including the entire L4 gene, whereas the primer pair L22-1f 5′-GGACGAATACTATA AGAGAAA-3′ and L22-4r 5′-TGTTGATACCTTGTAGCC-3′ amplifies a DNA fragment of 420 bp including the entire L22 gene. Target amplification was performed by an initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min and a final extension at 72°C for 5 min. PCR products were then sequenced and analysed for mutations by comparison with deposited sequences using the BLAST software.

Mutations in the gyrA and gyrB genes in C. difficile strains resistant to fluoroquinolones were detected as previously described. The quinolone resistance-determining region (QRDR) of both gyrA and gyrB genes was amplified using two different couples of primers, sequenced and analysed for mutations conferring resistance to moxifloxacin and other fluoroquinolones.

The rpoB mutations responsible for rifampicin resistance were identified as previously described. Primers CDCrpoB2F and CDCrpoB2R were used to amplify a 200 bp region of rpoB gene known to contain point mutations associated with resistance to rifampicin. The purified PCR products were then sequenced and analysed for mutations.

**Nucleotide sequence accession numbers**

The sequences of two different tet(W) gene variants reported in this paper have been submitted to the EMBL Nucleotide Sequence Database. The accession numbers are FR838948 for the tet(W) identified in three C. difficile strains of PCR ribotype 048 and FR838949 for the gene found in a C. difficile strain of PCR ribotype 012.

**Results**

**Resistance and multidrug resistance in C. difficile strains**

In total, 354 patients met the criteria for CDI. Seventy-nine percent were healthcare facility-associated cases, whereas 21% were community acquired. One hundred and forty-eight of the 316 strains isolated and analysed (47%) were resistant to at least one antibiotic. In particular, 12 were resistant to only one antibiotic, 54 to two antibiotics and 82 to at least three antibiotics and were defined as multidrug-resistant strains.

All strains resistant to moxifloxacin were also resistant to gatifloxacin, levofloxacin and ciprofloxacin, with MICs between 8 and ≥32 mg/L for gatifloxacin and ≥32 mg/L for both levofloxacin and ciprofloxacin. All C. difficile strains analysed in this study were susceptible to metronidazole and vancomycin, with MIC90 values of 0.125 mg/L and 0.75 mg/L, respectively.

The MIC50 for multiresistant strains was ≥256 mg/L for erythromycin and clindamycin, 2 mg/L for chloramphenicol, 16 mg/L for moxifloxacin, ≥32 mg/L for rifampicin and 0.047 mg/L for tetracycline, whereas the MIC90 was ≥256 mg/L for erythromycin and clindamycin, ≥32 mg/L for moxifloxacin and rifampicin, 48 mg/L for chloramphenicol and 24 mg/L for tetracycline.

Multidrug-resistant strains belonged to 11 different PCR ribotypes, predominantly 001 (39%), 017 (18%) and 012 (12%), and were observed in all countries except Great Britain, Switzerland, The Netherlands and Turkey (Table 1). Forty-eight percent (39/82) of multidrug-resistant isolates were resistant to four different classes of antibiotics: erythromycin, clindamycin, moxifloxacin and rifampicin.

**Resistance due to antibiotic resistance determinants located on transposons**

Ninety of the 316 (28%) C. difficile strains were erm(B)-positive and 75 of them, all resistant to erythromycin and clindamycin, were multidrug-resistant strains (Table 2).

Thirty-one erm(B)-positive clinical isolates, representative of different phenotypes and PCR ribotypes, were analysed for the genetic organization of the Erm(B) determinant. Twenty of

---

### Table 1. Characteristics of the multidrug-resistant C. difficile isolates identified in the study

<table>
<thead>
<tr>
<th>PCR ribotype (no. of strains)</th>
<th>Country (no. of strains)</th>
<th>ERY CLI TET</th>
<th>ERY CLI MXF</th>
<th>ERY CLI MXF RIF</th>
<th>ERY CLI MXF TET</th>
<th>ERY CLI RIF TET</th>
<th>ERY CL MXF RIF CHL</th>
<th>ERY CL MXF RIF TET CHL</th>
</tr>
</thead>
<tbody>
<tr>
<td>001 (32)</td>
<td>D (3); E (27); IRL (2)</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>012 (10)</td>
<td>F (1); D (3); GR (1); H (4); PL (1)</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>017 (15)</td>
<td>D (1); GR (2); IRL (2); PL (7); S (3)</td>
<td>7</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>020 (2)</td>
<td>I (1); S (1)</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>048 (9)</td>
<td>D (3); GR (1); H (5)</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>055 (1)</td>
<td>IRL (1)</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>068 (1)</td>
<td>IRL (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>071 (1)</td>
<td>H (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>078 (1)</td>
<td>B (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>126 (1)</td>
<td>F (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>168 (9)</td>
<td>D (7); I (1); IRL (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>82</td>
<td>82</td>
<td>6</td>
<td>24</td>
<td>39</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

B, Belgium; D, Germany; E, Spain; F, France; GR, Greece; H, Hungary; I, Italy; IRL, Ireland; PL, Poland; S, Sweden; CHL, chloramphenicol; CLI, clindamycin; ERY, erythromycin; MXF, moxifloxacin; RIF, rifampicin; TET, tetracycline.

MIC range for ERY, CHL, CLI, MXF, RIF, TET was 6–256, 1–256, 12–256, 0.5–32, ≤0.002–≥32 and 0.023–≥32 mg/L, respectively.
these selected strains were multidrug resistant. Seven different genetic organizations, E1–E7, have been described in *C. difficile* so far, and the E1 organization characterizes the reference strain *C. difficile* 630.12 In this study, 14 different organizations were observed and 10 (E8–E17) are new (Table 3). The majority of isolates showed an Erm(B) determinant type E4 (29%) or E15 (13%). These types included nine and three multidrug-resistant strains, respectively. No association between PCR ribotype and *erm* (B) genetic organization was observed.

All strains resistant to tetracycline were multidrug resistant and were positive for Tn5397 (data not shown). These strains clustered in PCR ribotype 012 (eight isolates) or PCR ribotype 048 (six isolates) (Table 2). Four strains, three belonging to PCR ribotype 048 and one to PCR ribotype 012, showed the co-presence of tet(M) and tet(W), a tet class recently observed in another *C. difficile* clinical isolate, *C. difficile* CDS.17 Strains of PCR ribotype 048 were isolated from the same hospital in Hungary, so they probably have a clonal origin. In fact, the tet(W) genes found in these isolates had the same nucleotide sequence (EMBL accession number FR838948), which shows 99% identity with the tet(W) previously found in *C. difficile* CDS.17 The tet(W) of the strain belonging to PCR ribotype 012

<table>
<thead>
<tr>
<th>PCR ribotype (no. of strains)</th>
<th>erm(B) positive</th>
<th>erm(B) negative</th>
<th>Types of substitution (no. of strains) in</th>
<th>MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tet</td>
<td>ERY</td>
<td>CLI</td>
<td>PCR ribotype (no. of strains)</td>
<td>References for elements with a similar arrangement</td>
</tr>
<tr>
<td>001 (32)</td>
<td>32</td>
<td>Thr-82 → Ile (32)</td>
<td>His-502 → Asn + Arg-505 → Lys (27)</td>
<td>≥256</td>
</tr>
<tr>
<td>012 (10)</td>
<td>10</td>
<td>Thr-82 → Ile (7)</td>
<td>His-502 → Asn + Arg-505 → Lys (7)</td>
<td>8a</td>
</tr>
<tr>
<td>017 (15)</td>
<td>8</td>
<td>Thr-82 → Ile (14)</td>
<td>Asp-426 → Val (1)</td>
<td>6a</td>
</tr>
<tr>
<td>020 (2)</td>
<td>2</td>
<td>Thr-82 → Ile (2)</td>
<td>His-502 → Asn + Arg-505 → Lys (1)</td>
<td>5</td>
</tr>
<tr>
<td>048 (9)</td>
<td>9</td>
<td>Thr-82 → Ile (5)</td>
<td>His-502 → Asn + Arg-505 → Lys (5)</td>
<td>6a</td>
</tr>
<tr>
<td>055 (1)</td>
<td>1</td>
<td>Thr-82 → Ile (1)</td>
<td>His-502 → Asn + Arg-505 → Lys (2)</td>
<td>11</td>
</tr>
<tr>
<td>068 (1)</td>
<td>1</td>
<td>Thr-82 → Ile (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>071 (1)</td>
<td>1</td>
<td></td>
<td>Asp-426 → Asn (1)</td>
<td></td>
</tr>
<tr>
<td>078 (1)</td>
<td>1</td>
<td>Thr-82 → Ile (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>126 (1)</td>
<td>1</td>
<td>Thr-82 → Ile (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>168 (9)</td>
<td>9</td>
<td>Thr-82 → Ile (9)</td>
<td>His-502 → Asn + Arg-505 → Lys (2)</td>
<td>11</td>
</tr>
</tbody>
</table>

| No. of strains | 75 | 7 | 73 | 2 | 51 | 14 | 11 |
| No. of strains positive for tet(M) and tndX and trpX | | | | | |

*One strain of PCR ribotype 012 and three strains of PCR ribotype 048 showed the co-presence of tet(M) and tet(W).
(EMBL accession number FR838949) shows 98% identity with both the gene of C. difficile CD5 and that of strains belonging to PCR ribotype 048. No amplifications of tet(W) flanking regions were obtained for the four strains analysed in this study. As observed for tetracycline, all 11 strains resistant to chloramphenicol were multidrug resistant (data not shown). These strains had an element belonging to the Tn4451/Tn4453 family, were positive for both cat(D) and trnpx and were typed as 012 (6 isolates) or 048 (5 isolates) (Table 2).

**Resistance due to modification of antibiotic targets**

Sequence analysis indicated that the majority of the isolates resistant to moxifloxacin had the amino acid substitution Thr→Ile in GyrA (data not shown). Seventy-three multidrug-resistant C. difficile strains showed the substitution Thr→Ile in GyrA, whereas two PCR ribotype 014 and 071 strains showed a substitution in position 426 of GyrB, Asp→426→Val and Asp→426→Asn, respectively (Table 2).

All strains resistant to rifampicin had one or two substitutions in RpoB (data not shown). In particular, 49/53 (92%) had two substitutions, His→502→Asn and Arg→505→Lys; 3/53 (6%) had only the substitution Arg→505→Lys and 1/53 (2%) had only the substitution His→502→Tyr. Among multidrug-resistant strains, 49 showed two substitutions, His→502→Asn and Arg→505→Lys, and 2 isolates had only the substitution Arg→505→Lys (Table 2).

All substitutions observed in GyrA, GyrB and RpoB have already been described in C. difficile resistant clinical isolates.22–24

**Investigations on resistance in erm(B)-negative strains**

Fifty-three C. difficile strains were erm(B) negative. In particular, nine of these strains were resistant to both erythromycin and clindamycin, whereas the majority (44/53) were resistant only to erythromycin. Seven of these, all PCR ribotype 017 isolated in different countries and hospitals, were multidrug resistant (Tables 1 and 2).

All erm(B)-negative strains were also negative for the other predominant erm genes found in anaerobes (A, C, E, F and Q), except for one strain showing an erm(Q) gene (data not shown). Nine resistant erm(B)-negative clinical isolates were subjected to the action of two efflux pump inhibitors, reserpine and CCCP, but they did not show any reduction in MICs (data not shown).

Since resistance to MLSB is also due to alteration of the binding site for these antibiotics,20,61 23 selected erm(B)-negative isolates and 17 susceptible strains, used as controls, were investigated for mutations in the 23S genes, but sequence analysis indicated the absence of mutations conferring resistance (data not shown). Interestingly, we observed the mutation C→T, previously described by Schmidt et al.,38 in position 656 of the 23S rDNA of 6 erm(B)-negative strains, in 4 strains resistant to both erythromycin and clindamycin and in 7 strains resistant only to erythromycin, but also in 2 of the 16 susceptible control strains (data not shown). Furthermore, 13 erm(B)-negative isolates, 6 erm(B)-positive strains and the susceptible control strains were also investigated for alterations in the ribosomal proteins L4 and L22 (data not shown). All C. difficile strains of PCR ribotype 126, erm(B) negative or positive, showed a variant L4 protein already observed in the PCR ribotype 078 C. difficile strain M120 (GenBank accession number FN665653, positions 112203–112856). The same variant was also found in one susceptible strain always belonging to PCR ribotype 078. This result is not surprising since PCR ribotypes 078 and 126 are closely related.42 The presence of the same L4 ribosomal protein variant in erm(B)-negative and in susceptible strains indicates that it is not involved in resistance to MLSB antibiotics. Two different L22 variant genes were identified, but these mutations do not determine amino acid changes in the encoded protein. The first variant was observed in all strains belonging to PCR ribotypes 126 and 078 and is identical to that found in C. difficile M120 (GenBank accession number FN665653, positions 114354–114689), the second was observed in three PCR ribotype 017 strains and is identical to that found in the PCR ribotype 017 C. difficile M68 (GenBank accession number FN668375, positions 76529–76864).

**Discussion**

This study provides a comprehensive analysis of the antibiotic resistance in 316 C. difficile clinical isolates collected in 14 different European countries during a prospective study performed in 2005.60

One hundred and forty-eight of the 316 strains analysed (47%) were resistant to at least one antibiotic and 55% of these (82/148) were multidrug resistant, showing resistance to at least three different classes of antibiotics. In particular, the majority were resistant to erythromycin, clindamycin, moxifloxacin and rifampicin. The multidrug-resistant strains belonged to 11 different PCR ribotypes, but the majority belonged to PCR ribotypes 001 (39%), 017 (18%) and 012 (12%). Previously a local epidemic strain belonging to PCR ribotype 012 was identified as multidrug resistant in a study conducted in Sweden.53

Resistance to erythromycin and clindamycin in C. difficile populations is long standing and in this study characterized all multidrug-resistant strains. As far as the resistance mechanism is concerned, 63% of all resistant strains were erm(B) positive, whereas 37% were erm(B) negative. In particular, all strains resistant to both erythromycin and clindamycin, except nine, were erm(B) positive, whereas all strains resistant only to erythromycin were erm(B) negative. Among multidrug-resistant strains, the erm(B)-negative strains were all typed as PCR ribotype 017 and were isolated in different countries and hospitals, suggesting a large circulation of a specific clone.

These results indicate that an erm(B) gene is still the most common determinant of resistance to MLSB antibiotics in C. difficile, although in some other studies, performed in a single hospital or in a geographically restricted area, a high percentage of erm(B)-negative strains, ranging between 56% and 61%, have been reported.3,38,64,65

As is already known, different elements carrying the Erm(B) resistance determinant have been found in C. difficile.12,66 The simplest is represented by a single erm(B) gene and the most complex by Tn5398, with its duplicated (erm(B) genes.11,66 In this study we identified 10 elements with new genetic organizations, and type E4 and E15 were the most detected in the multidrug-resistant strains. The great heterogeneity in the genetic arrangement of MLSB resistance determinants confirms
that genetic exchange and recombination frequently occur in clinical strains.

Further investigations, performed on the erm(B)-negative *C. difficile* strains identified in this study, showed that resistance was due neither to other erm classes usually found in resistant anaerobes nor to an overexpression of multidrug efflux pumps. The analysis of the 23S rDNA, the target site for the MLSβ antibiotics, showed the mutation C656 → T, previously observed by other authors, in some resistant erm(B)-negative isolates and also in some susceptible strains, suggesting that it cannot have any role in resistance. A variant of the L4, a ribosomal protein involved in the functional maintenance of the three-dimensional structure of the rRNA, was found in three erm(B)-negative strains, but also in one susceptible strain, supporting the exclusion of alterations in the target site as the cause of resistance in erm(B)-negative *C. difficile* strains. No variations in the L22 of erm(B)-negative resistant strains and erm(B)-positive strains were observed. Further studies are needed to identify the mechanisms of resistance harboured by these strains.

All strains resistant to tetracycline or chloramphenicol were multidrug resistant and belonged to PCR ribotype 012 or 048. In *C. difficile*, resistance to tetracycline is usually due to the presence of a tet(M) gene carried by the conjugative transposon Tn5397. Interestingly, four isolates showed the co-presence of tet(M) and tet(W). tet(W) has the second-largest host range and has often been detected in both Gram-positive and Gram-negative bacteria, especially in those isolated from environmental samples. The tet(W) genes found in this study are new variants and are carried by elements different from the one previously identified in the clinical isolate *C. difficile* CDS. The percentage of *C. difficile* strains resistant to chloramphenicol ranged between 14.6% and 26%. In this study, 7% of the isolates were resistant to this antibiotic and all harboured a Tn4451/Tn4453 transposon.

Resistance to moxifloxacin has increased dramatically over the last several years, and recent reports have described an association between fluoroquinolone exposure and CDI, particularly in outbreaks due to hypervirulent strains BI/NAP1/027. Resistance to rifampicin was very recently detected, and studies conducted from 2008 to 2010 in different countries showed that the percentage of *C. difficile* strains resistant to this antibiotic is variable, ranging between 6.3% and 36.8%. In the present paper, the majority of multidrug-resistant strains (51/82) were resistant to both moxifloxacin and rifampicin. Interestingly, in these strains, resistance to rifampicin was always associated with resistance to moxifloxacin. The amino acid substitution Thr-82 → Ile in GyrA and the contemporary presence of two amino acid changes, His-502 → Asn and Arg-505 → Lys, in RpoB were found to be responsible for resistance to moxifloxacin and rifampicin, respectively. The increasing number of *C. difficile* isolates resistant to these antibiotics suggests easy acquisition of resistance in vivo. The role of rifampicin use in the emergence of resistance in *C. difficile* has not been clearly defined. However, the increased number of resistant isolates to this antibiotic and the possibility that resistance to rifampicin correlated completely with resistance to the other components of the rifamycin class suggests careful and moderate use of this antibiotic in the treatment of CDI recurrences. The driving force of fluoroquinolones in the emergence of epidemic strains, in particular the PCR ribotype 027, is well known. The acquisition of this resistance is spreading in *C. difficile* clinical isolates, confirming the necessity for conscientious use of these antibiotics.

The emergence of reduced susceptibility to metronidazole has been reported in the UK and heteroresistance of some strains has been described in Spain. In this study we did not find strains with reduced susceptibility to metronidazole, even if this result could be due to repeated passages of isolates on culture plates before testing, storage at –70°C and/or use of the Etest method, which has been reported to underevaluate the MICs in comparison with the agar dilution method. Similarly, we did not observe strains with reduced susceptibility to vancomycin.

In conclusion, this study is the first comprehensive analysis of multidrug resistance in a large collection of European *C. difficile* strains and represents a precious source of data for future comparative studies. The results obtained indicate that multiresistance characterized the majority of resistant *C. difficile* strains in 2005, and some of these *C. difficile* types, such as PCR ribotypes 001, 012 and 017, are still frequently isolated from CDI in clinical settings, as observed in the second hospital-based survey of 2008. The high percentage of multidrug-resistant strains we have found leaves us to suppose that the acquisition and maintenance of antibiotic resistance are important not only in the emergence of epidemic clones, but also in the persistence of specific types over time in hospital settings, even if further analysis will be necessary to confirm this hypothesis. The mechanisms of resistance have also been investigated and the results indicate that antibiotic resistance in *C. difficile* is evolving. The capacity to acquire and disseminate resistance determinants from and to other species and to maintain genomic mutations conferring resistance to new classes of antibiotics provide *C. difficile* with potential advantages over the co-resident gut flora. Monitoring of both the resistance and emergence of highly virulent clones and better stewardship in the use of antibiotics are needed for efficient control of *C. difficile* infections.

**Acknowledgements**

We gratefully thank F. Barbut and E. Kuijper for a critical reading of the manuscript prior to submission. We also thank Tonino Sofia for editing the manuscript.

**Members of the European Study Group on Clostridium difficile (ESGCD)**


**Funding**

This work was partially supported by EC Project LSHE-CT-2006-037870, ’European approach to combat outbreaks of Clostridium difficile associated diarrhoea by development of new diagnostic tests’.

**Transparency declarations**

None to declare.
References

16. Roberts AP, Johanesen PA, Lyras D et al. Comparison of Tn5397 from Clostridium difficile, Tn916 from Enterococcus faecalis and the CW459 tet(M) element from Clostridium perfringens shows that they have similar conjugation regions but different insertion and excision modules. Microbiology 2001; 147: 1243–51.


