Fluoroquinolone resistance in *Clostridium difficile* isolates from a prospective study of *C. difficile* infections in Europe

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The European Study Group on *Clostridium difficile* (ESGCD) conducted a prospective study in 2005 to monitor and characterize *C. difficile* strains circulating in European hospitals, collecting 411 isolates. Eighty-three of these isolates, showing resistance or intermediate resistance to moxifloxacin (MX), were selected for this study to assess susceptibility to other fluoroquinolones (FQs) and to analyse the *gyr* genes, encoding the DNA gyrase subunits GyrA and GyrB. Twenty MX-susceptible isolates from the surveillance study were included for comparison. Overall, one amino acid substitution in GyrA (Thr82 to Ile) and four different substitutions in GyrB (Ser416 to Ala, Asp426 to Asn, Asp426 to Val and Arg447 to Lys) were identified. A high level of resistance (MIC ≥ 32 $\mu\text{g ml}^{-1}$) to MX, ciprofloxacin (CI), gatifloxacin (GA) and levofloxacin (LE) was found in 68 isolates showing the amino acid substitution Thr82 to Ile in GyrA, in eight isolates with the substitutions Thr82 to Ile in GyrA and Ser416 to Ala in GyrB, in two isolates showing the substitution Asp426 to Asn in GyrB and in one isolate with Asp426 to Val in GyrB. The remaining four isolates showed high MICs for CI and LE, but different MIC levels for MX and GA. In particular, intermediate levels of resistance to MX were shown by two isolates, one with the substitution Thr82 to Ile in GyrA, and one showing Asp426 to Asn in GyrB. The substitution Arg447 to Lys in GyrB was found in two strains resistant to MX, CI and LE but susceptible to GA. No substitutions in GyrA were found in the FQ-susceptible strains, whereas two strains showed the amino acid change Ser416 to Ala in GyrB. Thr82 to Ile was the most frequent amino acid change identified in the *C. difficile* isolates examined. In contrast to previous observations, 10% of the isolates showed this substitution in association with Ser416 to Ala in GyrB. The other amino acid changes found were characteristic of a few strains belonging to certain types and/or countries. Two new substitutions for *C. difficile*, Ser416 to Ala and Arg447 to Lys, were found in GyrB. Whereas the former does not seem to have a key role in resistance, since it was also detected in susceptible strains, the latter substitution occurred in the same position where other amino acid variations take place in resistant *Escherichia coli* and other *C. difficile* strains. A large number of *C. difficile* isolates now show an alarming pattern of resistance to the majority of FQs currently used in hospitals and outpatient settings, therefore judicious use of these antibiotics and continuous monitoring of *in vitro* resistance are necessary.

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Abbreviations: CI, ciprofloxacin; FQs, fluoroquinolones; GA, gatifloxacin; LE, levofloxacin; MX, moxifloxacin; QRDR, quinolone-resistance determining region. The GenBank/EMBL/DDBJ accession numbers for the five new sequences for *gyrA* and four for *gyrB* are AM890062–AM890070.

INTRODUCTION

Antibiotic treatment is one of the principal risk factors for *Clostridium difficile*-associated disease (CDAD). In the past, fluoroquinolones (FQs) were considered a low risk for CDAD (Golledge *et al.*, 1992), but recent studies have shown an association, in particular in recent outbreaks caused by the PCR ribotype 027/toxinotype III epidemic clone (Biller *et al.*, 2007; Gaynes *et al.*, 2004; McCusker *et al.*, 2003; Muto *et al.*, 2005; Yip *et al.*, 2001).

Because of their wide spectrum of activity, FQs have been extensively used in clinical medicine against both Gram-negative and Gram-positive bacteria. FQs act by inhibiting the action of type II topoisomerases, DNA gyrase and topoisomerase IV, essential for bacterial DNA replication (Hooper, 1999). Two main mechanisms of quinolone resistance have been identified: alterations in the target enzymes, widely spread in many bacteria; and decreased antibiotic accumulation inside the bacterium due to impermeability of the membrane and/or an overexpression of efflux pump systems (Ruiz, 2003). In the first mechanism, resistance is due to amino acid substitutions, particularly to those occurring in a certain region of the enzyme subunit called the quinolone-resistance determining region (QRDR), which makes the enzyme less sensitive to inhibition by FQs (Ruiz, 2003).

Analysis of the *C. difficile* genome has demonstrated that this bacterium lacks genes for topoisomerase IV, as already observed in other species such as *Treponema pallidum*, *Mycobacterium tuberculosis* and *Helicobacter pylori* (Dridi *et al.*, 2002). FQ-resistant *C. difficile* clinical isolates analysed so far have shown alterations in the QRDR of either GyrA or GyrB (Ackermann *et al.*, 2001, 2003; Dridi *et al.*, 2002; Drudy *et al.*, 2006, 2007).

The European Study Group on *C. difficile* (ESGCD) conducted a prospective study from April to June 2005 to monitor CDAD and characterize a large sample of *C. difficile* strains circulating in European hospitals (Barbut *et al.*, 2007). The 411 isolates collected were tested in our laboratory for their susceptibility to different antibiotics, including moxifloxacin (MX). In the present study, we selected and analysed 83 of those isolates that showed resistance or intermediate resistance to MX, in order to obtain a more detailed picture of their FQ resistance and to identify the related mutations in *gyr* genes.

METHODS

***C. difficile* isolates.** Four hundred and eleven isolates were collected from 38 different hospitals in 14 countries during the European collaborative study (Barbut *et al.*, 2007). The strains were typed by toxinotyping (Rupnik *et al.*, 1998) and PCR ribotyping (Stubbs *et al.*, 1999), and analysed for antibiotic resistance. Thirty-three per cent (134/411) showed intermediate susceptibility or resistance to MX. In particular, two isolates showed MICs ≥ 2 – <8 $\mu\text{g ml}^{-1}$ and 132 showed MICs ≥ 8 $\mu\text{g ml}^{-1}$. One hundred and thirty of the 134

isolates were toxigenic, whereas four were non-toxigenic. Eighty-three of the 134 isolates (62 %), resistant or intermediate to MX, were selected for this study (Table 1) as representative of each country of origin, toxinotype/PCR ribotype and MX phenotype (intermediate, MIC ≥ 4 – <8 $\mu\text{g ml}^{-1}$; resistant, MIC ≥ 8 – <32 $\mu\text{g ml}^{-1}$; highly resistant, MIC ≥ 32 $\mu\text{g ml}^{-1}$) (CLSI, 2007). In total, 79 (61 %) of the 130 toxigenic strains, including all PCR ribotype 027/toxinotype III strains (11 in Belgium, 8 in the Netherlands and 1 in Ireland) and the four non-toxigenic strains, were included in the study. Twenty MX-susceptible strains, selected by the criteria mentioned above, were analysed as controls.

PCR ribotyping. During the European collaborative study, only toxigenic strains were typed by PCR ribotyping (Barbut *et al.*, 2007) using the method described by Stubbs *et al.* (1999). In the present study, four non-toxigenic strains resistant to MX were also PCR-ribotyped in the Anaerobe Reference Laboratory, University Hospital of Wales, Cardiff, UK.

Antibiotic susceptibility. MIC values for MX, ciprofloxacin (CI), gatifloxacin (GA) and levofloxacin (LE) were determined by the E-test (AB biodisk) on *Brucella* agar plates containing vitamin K₁ (0.5 mg l⁻¹), haemin (5 mg l⁻¹) and 5 % defibrinated sheep red blood cells, according to the manufacturer's instructions. The breakpoint used

Table 1. The 83 *C. difficile* isolates selected for the study

Country	Toxigenic status	<i>C. difficile</i> isolates from the European Study	
		With a MIC ≥ 4 $\mu\text{g ml}^{-1}$ for MX	Analysed in this study
Belgium	Toxigenic	13	12
	Non-toxigenic	0	0
Switzerland	Toxigenic	1	1
	Non-toxigenic	0	0
Germany	Toxigenic	38	12
	Non-toxigenic	0	0
Spain	Toxigenic	30	12
	Non-toxigenic	0	0
France	Toxigenic	4	4
	Non-toxigenic	0	0
Great Britain	Toxigenic	7	4
	Non-toxigenic	0	0
Greece	Toxigenic	6	4
	Non-toxigenic	1	1
Hungary	Toxigenic	10	4
	Non-toxigenic	1	1
Italy	Toxigenic	4	3
	Non-toxigenic	2	2
The Netherlands	Toxigenic	13	8
	Non-toxigenic	0	0
Ireland	Toxigenic	20	9
	Non-toxigenic	0	0
Poland	Toxigenic	10	5
	Non-toxigenic	0	0
Sweden	Toxigenic	3	1
	Non-toxigenic	0	0

was 8 mg l⁻¹ (CLSI, 2007). *Bacteroides thetaiotaomicron* ATCC 29741 was tested as a quality control strain.

Amplification and sequencing of *gyr* genes. The QRDRs of *gyrA* and *gyrB* were amplified using the primer couple *gyrA*1 (5'-AATGAGTGTATAGCTGGACG-3') and *gyrA*2 (5'-TCT TTT AAC GAC TCA TCA AAG TT-3'), amplifying 390 bp of *gyrA*, and the primer couple *gyrB*1 (5'-AGT TGA TGA ACT GGG GTC TT-3') and *gyrB*2 (5'-TCA AAA TCT TCT CCA ATA CCA-3'), amplifying 390 bp of *gyrB*, as described by Dridi *et al.* (2002). PCR products were purified using the NucleoSpin Extract kit (Macherey-Nagel) and sequenced by the Big Dye Terminator v.1.1 Cycle Sequencing kit (Applied Biosystems) and an Applied Biosystems 3730 DNA Analyser. Sequences were compared using the BLAST server of the National Center for Biotechnology Information.

RESULTS AND DISCUSSION

Molecular typing of *C. difficile*

The 79 toxigenic strains analysed in this study belonged to five toxinotypes (0, I, III, V and VIII) and to 19 different PCR ribotypes (Table 2). Forty-eight per cent of the isolates belonged to toxinotype 0, whereas the others were toxin variant strains. In particular, eight strains belonged to toxinotype V, 12 to toxinotype VIII (which usually groups toxin A-negative/toxin B-positive strains; Rupnik *et al.*, 1998) and only one to toxinotype I. The toxinotype III group contained all 20 *C. difficile* isolates characterized as PCR ribotype 027, as already observed in other studies

Table 2. Phenotypic and genotypic characteristics of the 79 toxigenic and 4 non-toxicogenic FQ-resistant *C. difficile* isolates analysed in the study

Toxinotype (n)	PCR ribotype (n)	MIC (µg ml ⁻¹) or MIC range for:*				Amino acid substitutions in:		Country (n)†
		MX	CI	GA	LE	GyrA	GyrB	
0 (38)	001 (13)	≥32	≥32	≥32	≥32	Thr82→Ile		D(2) IRL(1) E(2) GB(1)
		≥8 <32	≥32	≥32	≥32	Thr82→Ile		D(1) IRL(1) E(5)
	012 (5)	≥8 <32	≥32	≥32	≥32	Thr82→Ile		F(1) D(2)
		≥32	≥32	≥32	≥32	Thr82→Ile		F(1) H(1)
	014 (2)	16	≥32	≥32	≥32	Thr82→Ile		GB(1)
		≥32	≥32	≥32	≥32		Asp426→Asn	CH(1)
	020 (3)	12	≥32	≥32	≥32	Thr82→Ile		I(1) E(1)
		≥32	≥32	≥32	≥32	Thr82→Ile		E(1)
	022 (1)	12	≥32	≥32	≥32	Thr82→Ile		E(1)
	048 (3)	12	≥32	≥32	≥32	Thr82→Ile		D(1) H(1)
		6	≥32	8	≥32		Asp426→Asn	H(1)
	055 (1)	12	≥32	≥32	≥32	Thr82→Ile		IRL(1)
	068 (1)	≥32	≥32	≥32	≥32	Thr82→Ile		IRL(1)
	077 (1)	16	≥32	≥32	≥32	Thr82→Ile		GB(1)
	106 (1)	≥32	≥32	≥32	≥32	Thr82→Ile		GB(1)
	156 (1)	12	≥32	≥32	≥32	Thr82→Ile		IRL(1)
I (1)	001 (1)	≥32	≥32	≥32	≥32	Thr82→Ile		D(3) I(1) IRL(1)
		16	≥32	≥32	≥32	Thr82→Ile		D(1)
III (20)	027 (20)	≥32	≥32	≥32	≥32	Thr82→Ile		E(1)
		≥8 <32	≥32	≥32	≥32	Thr82→Ile		B(6) NL(3)
		6	≥32	≥32	≥32	Thr82→Ile		B(4) NL(5) IRL(1)
V (8)	078 (2)	8	≥32	≥32	≥32	Thr82→Ile	Ser416→Ala	B(1) GR(1)
		12	≥32	≥32	≥32	Thr82→Ile	Ser416→Ala	I(1)
		≥8 <32	≥32	≥32	≥32	Thr82→Ile	Ser416→Ala	F(2) D(1) GR(1) E(1)
VIII (12)	017 (11)	≥32	≥32	≥32	≥32	Thr82→Ile		D(1) IRL(1) PL(5) S(1)
		≥32	≥32	≥32	≥32		Asp426→Val	GR(2)
	071 (1)	8	≥32	16	≥32		Asp426→Asn	IRL(1)
	010 (1)	12	≥32	3	≥32		Arg447→Lys	H(1)
	010 (2)	8	≥32	3	≥32		Arg447→Lys	I(1)
Non-toxicogenic		≥32	≥32	≥32	≥32	Thr82→Ile		I(1)
Non-toxicogenic	039 (1)	≥32	≥32	≥32	≥32	Thr82→Ile		H(1)
Non-toxicogenic		≥32	≥32	≥32	≥32	Thr82→Ile		GR(1)

*MX, Moxifloxacin; CI, ciprofloxacin; GA, gatifloxacin, LE, levofloxacin.

†B, Belgium; CH, Switzerland; D, Germany; E, Spain; F, France; GB, Great Britain; GR, Greece; H, Hungary; I, Italy; NL, the Netherlands; IRL, Ireland; PL, Poland; S, Sweden.

(Kuijper *et al.*, 2006). The non-toxigenic strains belonged to PCR ribotypes 010 and 039, different from those of toxigenic strains (Table 2). Thirteen of the 20 selected MX-susceptible isolates belonged to toxinotype 0, two to toxinotype V and one to toxinotype III. The other four strains were non-toxigenic (data not shown).

Susceptibility to FQs

MIC values ($\mu\text{g ml}^{-1}$) for the selected *C. difficile* isolates are shown in Table 2. The MIC range was 6– ≥ 32 for MX, 3– ≥ 32 for GA and ≥ 32 for both CI and LE. MIC₅₀ and MIC₉₀ values for MX were 16 and $\geq 32 \mu\text{g ml}^{-1}$, respectively, and $\geq 32 \mu\text{g ml}^{-1}$ for CI, GA and LE. Interestingly, all strains were fully resistant to CI and LE, including the two isolates with an intermediate MIC level for MX (MIC=6 $\mu\text{g ml}^{-1}$) and the two non-toxigenic isolates susceptible to GA (MIC=3 $\mu\text{g ml}^{-1}$). All strains susceptible to MX were also susceptible to the other FQs tested, except for one strain with MIC=6 $\mu\text{g ml}^{-1}$ for CI (data not shown).

gyrA and *gyrB* sequence analysis

The sequence analysis of both *gyrA* and *gyrB* indicated that 83 % of the *C. difficile* isolates analysed (69/83) had a nucleotide mutation leading to a single amino acid substitution in GyrA, 10 % (8/83) had an amino acid substitution in both GyrA and GyrB and 7 % (6/83) had a single amino acid change in GyrB (Table 2). No substitutions in GyrA were found in the *C. difficile* susceptible isolates, whereas two of these strains showed an amino acid substitution in GyrB (data not shown).

Overall, one substitution in GyrA (Thr82 to Ile) and four different substitutions in GyrB (Asp426 to Asn, Asp426 to Val, Ser416 to Ala and Arg447 to Lys) were identified. Thr82 to Ile was found in different *C. difficile* types and was characteristic of the majority of the isolates. It was also detected in all PCR ribotype 027/toxinotype III strains isolated in the European study, confirming previous data on such strains from Ireland (Drudy *et al.*, 2007). In contrast to other observations (Ackermann *et al.*, 2001, 2003; Dridi *et al.*, 2002; Drudy *et al.*, 2007), Thr82 to Ile was found in eight strains belonging to toxinotype V in association with Ser416 to Ala in GyrB.

The substitutions found in GyrB seemed to be characteristic of certain *C. difficile* types and countries. In particular, Asp426 to Asn in GyrB was found only in toxinotype 0 (one isolate from Switzerland and one from Hungary; PCR ribotypes 014 and 048, respectively) and VIII (one strain from Hungary; PCR ribotype 071). The substitution Asp426 to Val was identified only in one Irish isolate belonging to toxinotype VIII, as already reported by Drudy *et al.* (2006). The amino acid change Ser416 to Ala (GyrB) was found only in strains clustered in toxinotype V and Arg447 to Lys was found in two non-toxigenic strains, isolated in Italy. The substitutions Asp426 to Asn or Val

and Arg447 to Lys in GyrB were not associated with other amino acid changes.

Thr82 corresponds to Ser83 in *Escherichia coli* and substitutions of amino acid in equivalent position to Ser83 have been demonstrated to cause resistance to quinolones in many bacterial species (Hooper, 1999). This substitution was previously described in *C. difficile* by other authors (Ackermann *et al.*, 2001, 2003; Dridi *et al.*, 2002; Drudy *et al.*, 2007). The substitution Asp426 to Asn has already been found in resistant strains of *E. coli* (Hooper, 1999) and *C. difficile* (Dridi *et al.*, 2002), whereas the substitution Asp426 to Val has been described only in toxin A-negative, toxin B-positive *C. difficile* strains (Drudy *et al.*, 2006). The substitution Ser416 to Ala has never been described in any other bacteria resistant to FQs. Furthermore, in this study, it was also found in two MX-susceptible isolates belonging to toxinotype V (data not shown): one was fully susceptible to all FQs tested; the second showed an intermediate level of resistance to CI (MIC=6 $\mu\text{g ml}^{-1}$). Further studies will be necessary to better understand whether this substitution plays any role in resistance. In contrast, Arg447 to Lys occurred in the same position where other amino acid variations take place in FQ-resistant *E. coli* (Hooper, 1999) and *C. difficile* (Dridi *et al.*, 2002) strains. This substitution was not found in any susceptible *C. difficile* strain examined.

Eight different partial sequences for *gyrA* and seven for *gyrB* were identified (data not shown). These sequences differentiated nucleotide changes leading to amino acid changes and/or to silent mutations. Six partial sequences were previously described by Drudy *et al.* (2006, 2007), with accession numbers DQ821481, DQ821482, DQ821483, DQ642011, DQ642012 and DQ642013. In this study, five new sequences for *gyrA* and four for *gyrB* were submitted to EMBL and were assigned the accession numbers AM890062, AM890063, AM890064, AM890065, AM890066, AM890067, AM890068, AM890069 and AM890070.

FQ susceptibility patterns and genotypic characteristics

Different patterns of FQ susceptibility were identified (Table 3). A high level of resistance (MIC $\geq 32 \mu\text{g ml}^{-1}$) to all the FQs tested in the study was found in association with the amino acid substitution Thr82 to Ile in GyrA (38 strains) and Asp426 to Asn in GyrB (one strain). Fifty-five per cent (38/69) of the isolates with Thr82 to Ile in GyrA had a high level of resistance (MIC $\geq 32 \mu\text{g ml}^{-1}$) to MX, 43 % (30/69) had a lower level of resistance (MIC ≥ 8 – $< 32 \mu\text{g ml}^{-1}$) and 2 % (1/69) had an intermediate level of resistance. Similarly, Asp426 to Asn was found in one isolate highly resistant to MX, in one isolate resistant to this antibiotic (MIC=8 $\mu\text{g ml}^{-1}$) and in another showing an intermediate MIC level (MIC=6 $\mu\text{g ml}^{-1}$). Further analysis should be performed to verify whether the different phenotypes associated with the same amino acid

Table 3. FQ susceptibility patterns and amino acid substitutions of the 83 *C. difficile* isolates analysed in the study

FQ susceptibility patterns*				Amino acid substitutions in:		No. of <i>C. difficile</i> isolates
MX	CI	GA	LE	GyrA	GyrB	
HR	HR	HR	HR	Thr82→Ile		38
					Asp426→Asn	1
					Asp426→Val	1
R	HR	HR	HR	Thr82→Ile		30
				Thr82→Ile	Ser416→Ala	8
I	HR	HR	HR	Thr82→Ile		1
R	HR	R	HR		Asp426→Asn	1
I	HR	R	HR		Asp426→Asn	1
R	HR	S	HR		Arg447→Lys	2

*MX, moxifloxacin; CI, ciprofloxacin; GA, gatifloxacin; LE, levofloxacin; HR, highly resistant ($\text{MIC} \geq 32 \mu\text{g ml}^{-1}$); R, resistant ($\text{MIC} \geq 8\text{--}<32 \mu\text{g ml}^{-1}$); I, intermediate ($\text{MIC} \geq 4\text{--}<8 \mu\text{g ml}^{-1}$); S, sensitive.

substitution may be due to the presence of other mechanisms of resistance and/or to amino acid changes outside the *gyrA* and *gyrB* QRDR, extending the DNA region involved in the resistance, as proposed for other bacteria (Friedman *et al.*, 2001).

Interestingly, the substitution Arg447 to Lys was associated with resistance to MX, CI and LE and with susceptibility to GA. However, the small number of strains analysed with these characteristics does not permit any general conclusion. As in other bacteria, *gyrB* mutations in *C. difficile* occurred less commonly than *gyrA* mutations and tended to confer lower levels of resistance (Hooper, 1999).

In summary, resistance to FQs and the presence of amino acid substitutions in both GyrA and GyrB were analysed in 79 toxigenic and 4 non-toxigenic *C. difficile* isolates collected during the European collaborative study performed in 2005. Overall, the results indicated an alarming pattern of FQ resistance in *C. difficile* circulating in European hospitals, as already observed for many other human pathogens. Careful and continuous monitoring of FQ resistance and judicious use of these antibiotics is necessary to reduce the spread of resistant strains and the risk of diseases and outbreaks associated with *C. difficile*.

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