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Molecular Analysis of the *gyrA* and *gyrB* Quinolone Resistance-Determining Regions of Fluoroquinolone-Resistant *Clostridium difficile* Mutants Selected In Vitro[▽]

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Recent studies have suggested that exposure to fluoroquinolones represents a risk factor for the development of *Clostridium difficile* infections and that the acquisition of resistance to the newer fluoroquinolones is the major reason facilitating wide dissemination. In particular, moxifloxacin (MX) and levofloxacin (LE) have been recently associated with outbreaks caused by the *C. difficile* toxinotype III/PCR ribotype 027/pulsed-field gel electrophoresis type NAP1 strain. In this study, we evaluated the potential of MX and LE in the in vitro development of fluoroquinolone resistance mediated by GyrA and GyrB alterations. Resistant mutants were obtained from five *C. difficile* parent strains, susceptible to MX, LE, and gatifloxacin (GA) and belonging to different toxinotypes, by selection in the presence of increasing concentrations of MX and LE. Stable mutants showing substitutions in GyrA and/or GyrB were obtained from the parent strains after selection by both antibiotics. Mutants had MICs ranging from 8 to 128 μg/ml for MX, from 8 to 256 μg/ml for LE, and from 1.5 to ≥32 μg/ml for GA. The frequency of mutation ranged from 3.8×10^{-6} to 6.6×10^{-5} for MX and from 1.0×10^{-6} to 2.4×10^{-5} for LE. In total, six different substitutions in GyrA and five in GyrB were observed in this study. The majority of these substitutions has already been described for clinical isolates or has occurred at positions known to be involved in fluoroquinolone resistance. In particular, the substitution Thr82 to Ile in GyrA, the most common found in resistant *C. difficile* clinical isolates, was observed after selection with LE, whereas the substitution Asp426 to Val in GyrB, recently described in toxin A-negative/toxin B-positive epidemic strains, was observed after selection with MX. Interestingly, a reduced susceptibility to fluoroquinolones was observed in colonies isolated after the first and second steps of selection by both MX and LE, with no substitution in GyrA or GyrB. The results suggest a relevant role of fluoroquinolones in the emergence and selection of fluoroquinolone-resistant *C. difficile* strains also in vivo.

Recent outbreaks of *Clostridium difficile* infections (CDI), with increased severity, high relapse rates, and significant mortality, have been related to the emergence of the hypervirulent *C. difficile* clone toxinotype III/PCR ribotype 027/pulsed-field gel electrophoresis type NAP1 (5, 23, 25–29, 31). Several studies have suggested that exposure to fluoroquinolones represents a risk factor for the development of CDI caused by *C. difficile* III/027/NAP1 and that the acquisition of resistance to the newer fluoroquinolones could have promoted its wide dissemination (6, 17, 30, 32–34).

Fluoroquinolones are a family of broad-spectrum antibiotics extensively used in the treatment of a great variety of human infections. The in vitro activity of the older fluoroquinolones, such as ciprofloxacin, has been reported to be moderate or poor against anaerobes, including *C. difficile* (3, 8), whereas the third and the fourth generations of fluoroquinolones are characterized by improved activity against gram-positive cocci and anaerobic bacteria (19, 36). Fluoroquinolones act by inhibiting the action of DNA gyrase and topoisomerase IV, which are related but distinct enzymes involved in DNA synthesis (18). The mechanisms of resistance to fluoroquinolones in bacteria are basically two: (i) alterations in the targets of fluoroquinolones and (ii) decreased accumulation inside the bacteria due to impermeability of the membrane and/or an overexpression of efflux pump systems (19, 20, 36). The first mechanism of resistance is widespread in many bacteria, and it is due to amino acid substitutions in the quinolone-resistance determining region (QRDR) of the target enzymes (35). This is the principal mechanism of resistance also in *C. difficile*, and since, as already observed in other species, this bacterium does not have genes for topoisomerase IV, resistance is determined by alterations in the QRDR of either DNA gyrase subunit GyrA or GyrB (10, 38).

Different amino acid substitutions have been identified in GyrA and GyrB in fluoroquinolone-resistant *C. difficile* strains. The most frequent is the amino acid change Thr82 to Ile in GyrA, which also characterizes the epidemic clone III/027/NAP1 (11, 38). Two other GyrA substitutions, Asp71 to Val and Ala118 to Thr, have been more rarely observed (1, 2, 10, 12, 38). Four different amino acid substitutions have been identified in GyrB: Arg447 to Lys, Arg447 to Leu, Asp426 to Asn, and Asp426 to Val (10, 11, 38). In particular, Asp426 to Val has been described in toxin A-negative/toxin B-positive *C. difficile* epidemic strains of recent isolation (11).

In this study, we evaluated the potential of moxifloxacin

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TABLE 1. Phenotypic and genotypic characteristics of *C. difficile* parent strains used in the study

Parent strain	Presence of *tcdA* and *tcdB*	Toxinotype	MIC (μg/ml)			Substitution in:	
			Agar dilution method for:		Etest for GA		
			MX	LE		GyrA	GyrB
C253	+	0	1	2	0.5		
CD5	+	V	1	2	0.5		Ser416 to Ala[a]
A422	+	III[b]	1	2	0.75		
630	+	0	1	2	0.75		
BI2 (nontoxigenic)	−		1	2	0.75		

[a] Observed in susceptible strains.
[b] PCR ribotype 027.

(MX) and levofloxacin (LE), recently associated with outbreaks caused by *C. difficile* III/027/NAP1 (25, 31, 33), for the in vitro development of fluoroquinolone resistance mediated by GyrA and GyrB alterations in five different susceptible *C. difficile* strains. The sequence changes occurring in the QRDR of the derived fluoroquinolone-resistant mutants were analyzed and correlated with the in vitro resistance to MX, LE, and gatifloxacin (GA), another fluoroquinolone recently involved in *C. difficile* outbreaks (17, 33).

MATERIALS AND METHODS

***C. difficile* isolates used for in vitro generation of resistance.** Four *C. difficile* clinical isolates of different toxinotypes and one nontoxigenic strain with comparable susceptibilities to MX, GA, and LE were selected for this study (Table 1). The *C. difficile* Italian strain C253 and the reference strain 630 belong to toxinotype 0 and were isolated at the beginning of the 1980s from hospital patients with severe CDI. CD5 is an Italian clinical isolate belonging to toxinotype V, isolated in 1998 from a symptomatic hospitalized child. *C. difficile* A422 is characterized as toxinotype III/PCR ribotype 027/pulsed-field gel electrophoresis type NAP1 and was isolated in Calgary, Alberta, Canada, in 2001. The nontoxigenic strain BI2 was isolated in France during the prospective study conducted by the European Study Group on *Clostridium difficile* (ESGCD) to monitor and characterize *C. difficile* strains circulating in European hospitals in 2005 (4).

Antibiotic susceptibility studies. The MICs for MX and LE were determined by the agar dilution method in brucella agar supplemented with vitamin K1 (0.5 mg/liter), hemin (5 mg/liter), and 5% defibrinated sheep red blood cells, as recommended by the Clinical and Laboratory Standards Institute (9), using pure MX powder, kindly supplied by Bayer HealthCare AG (Leverkusen, Germany), and LE powder (Sigma Chemical Co., St. Louis, MO). Susceptibility to GA was determined using an Etest (AB Biodisk, Solna, Sweden) by following the manufacturer's instructions. The breakpoint used for all fluoroquinolones tested in the study was 8 μg/ml (9).

Reserpine- and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)-sensitive efflux was tested by using MX or LE agar dilution in the presence and absence of these two potent inhibitors. In particular, reserpine has been shown to inhibit members of the major facilitator family of transporters, whereas CCCP has an effect on pumps that use the proton motive force for energy. Four sets of brucella agar-supplemented plates were made with doubling dilutions of the appropriate antibiotic. One set was used without inhibitors, whereas 20 or 80 μg/ml of reserpine (Sigma Chemical Co., St. Louis, MO) or 100 μM of CCCP (Sigma Chemical Co., St. Louis, MO) was added to the other three sets of plates, respectively. *C. difficile* strains with a fourfold or greater reduction in their MICs in the presence of inhibitors were considered positive for reserpine- and CCCP-sensitive efflux.

Selection of resistant mutants. *C. difficile* parent strains were grown in antibiotic-free brain heart infusion medium at 35°C in an anaerobic cabinet for 24 h. One hundred microliters of a 0.5 McFarland dilution (3×10^8 CFU/ml) of the brain heart infusion culture was used to inoculate Mueller-Hinton agar plates containing MX or LE at a concentration of 2 μg/ml or 4 μg/ml, respectively (step I). The inoculated plates were incubated under anaerobic conditions and examined for growth after 48 h. Three colonies were randomly picked up and subjected to MIC determination, sequence analysis, and a further step of selection on plates containing a double concentration of MX or LE compared to the MIC level (steps II and III). The stability of the mutant strains was assessed by three subcultures on Mueller-Hinton agar with and without MX and LE. Stable mutants were stored at −70°C and denominated with the name of the parent strains followed by a letter; a number-letter was used in the case of different mutants derived from the same parent strains. The frequency of mutation was calculated at the first step of selection, at which we obtained mutants for *gyrA* or *gyrB* as the number of colonies appearing on the plates divided by the number of bacteria in the inocula, enumerated by serial dilutions.

Amplification of the *gyrA* and *gyrB* QRDRs and sequencing. The *gyrA* and *gyrB* QRDRs of both the parent and mutant strains were amplified using the primer couple gyrA1 (5′-AATGAGTGTTATAGCTGGACG-3′) and gyrA2 (5′-TCT TTT AAC GAC TCA TCA AAG TT-3′), amplifying 390 bp of *gyrA*, and the primer couple gyrB1 (5′-AGT TGA TGA ACT GGG GTC TT-3′) and gyrB2 (5′-TCA AAA TCT TCT CCA ATA CCA-3′), amplifying 390 bp of *gyrB*, as already described (10).

DNA extraction was performed by boiling two to three colonies in 100 μl of distilled water for 10 min. PCR amplification consisted of 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C (*gyrB*)/58°C (*gyrA*) for 30 s, and extension at 72°C for 30 s.

The PCR products were purified using the NucleoSpin extract kit (Macherey-Nagel, Düren, Germany) and sequenced by the BigDye Terminator v.1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and the Applied Biosystems 3730 DNA analyzer. Pairwise alignments of DNA sequences were carried out using the BLAST server of the National Center for Biotechnology Information.

Nucleotide sequence accession numbers. The nucleotide sequences described in this paper have been deposited in the EMBL database with the accession numbers FM208112, FM208113, FM208114, FM208115, FM208116, FM208117, and FM208118 for the mutants selected in the presence of MX and FM999260, FM999261, FM999262, and FM999263 for the mutants selected in the presence of LE.

RESULTS

Characterization of *C. difficile* parent strains. The phenotypic and genotypic characteristics of the parent strains are reported in Table 1. All parent strains were susceptible to MX (MIC = 1 μg/ml), LE (MIC = 2 μg/ml), and GA (MICs between 0.5 and 0.75 μg/ml). No amino acid changes were found in GyrA and GyrB in these strains, except for CD5, which showed the amino acid substitution Ser416 to Ala in GyrB. As already observed in a previous paper (38), this substitution was found in other fluoroquinolone-susceptible strains, and thus, it is not associated with resistance.

Characterization of *C. difficile* mutants selected by MX. Stable mutants showing Gyr substitutions were obtained from all *C. difficile* parent strains used in this study. The frequency of mutation, calculated for the first step at which we detected mutations in the *gyr* genes, was 4.2×10^{-6} for C253, 3.8×10^{-6}

TABLE 2. Phenotypic and genotypic characteristics of *C. difficile* mutants obtained after selection by MX

Parent strain	Mutants selected	Step of selection (μg/ml MX)	MIC (μg/ml)			Substitution(s) in:	
			Agar dilution method for:		Etest for GA		
			MX	LE		GyrA	GyrB
C253	C253 a, b, c	I (2)	2	4	1		
	C253 d, e, f	II (4)	4	4	1		
	C253 g, h, i	III (8)	16	64	8	Ala118 to Ser	
CD5	CD5 a, b, c	I (2)	2	2	0.75		Ser416 to Ala
	CD5 d, e, f	II (4)	4	2	0.75		Ser416 to Ala
	CD5 1-g, h, i	III (8)	8	16	3	Ala92 to Glu	Ser416 to Ala
	CD5 2-g, h, i	III (8)	16	64	16	Ala118 to Ser/Thr82 to Ala	Ser416 to Ala
A422	A422 a, b, c	I (2)	2	4	1.5		
	A422 d, e, f	II (4)	4	4	1.5		
	A422 g, h, i	III (8)	32	64	≥32	Asp81 to Asn	
630	630 a, b, c	I (2)	2	4	0.75		
	630 d, e, f	II (4)	4	4	2		
	630 g, h, i	III (8)	16	64	≥32		Asp426 to Val
BI2	BI2 a, b, c	I (2)	16	8	1.5		Arg447 to Lys
	BI2 d, e, f	II (32)	128	64	6		Arg447 to Lys/Asp426 to Asn

for CD5, 5.4×10^{-6} for A422, 6.6×10^{-5} for 630, and 6.2×10^{-6} for the BI2 mutants.

In total, 45 colonies were isolated and their phenotypic and genotypic characteristics are shown in Table 2. The MICs ranged from 2 to 128 μg/ml for MX, from 2 to 64 μg/ml for LE, and from 0.75 to ≥32 μg/ml for GA. High levels of resistance were reached only after the acquisition of an amino acid change in GyrA or GyrB. The substitutions were detected at step I (2 μg/ml) for BI2 and at step III (8 μg/ml) for the other four parent strains.

Sequence analysis demonstrated the presence of amino acid changes in GyrA in mutants derived from *C. difficile* C253, CD5, and A422 and in GyrB in those from 630 and BI2. In particular, C253 mutants were characterized by MICs of 16 μg/ml for MX, 64 μg/ml for LE, and 8 μg/ml for GA and by the substitution Ala to Ser at position 118 of GyrA. This position corresponds to position 119 in *Escherichia coli*, and although located outside the QRDR (originally defined for this bacterium as being from amino acids 67 to 106), it has been recognized as responsible for fluoroquinolone resistance in numerous strains of *Salmonella* species (13, 21), suggesting a revision of the QRDR to include more amino acids as already proposed for amino acid 51 in *E. coli* (16).

Two different groups of mutants with different MICs and amino acid substitutions were obtained from *C. difficile* CD5. The first group had MICs of 8 μg/ml for MX, 16 μg/ml for LE, and 3 μg/ml for GA. This group of mutants was characterized by the substitution Ala to Glu at position 92 of GyrA, which corresponds to position 93 in *E. coli*. This substitution occurred in a position having a role in fluoroquinolone resistance, as demonstrated in *E. coli* (7, 39), and implies a change from a small, nonpolar, neutral amino acid to a larger-in-size, polar, negatively charged amino acid probably able to alter the shape of the drug-binding pocket and to consequently increase the MIC levels. The second group of CD5 mutants was characterized by MICs of 16 μg/ml for MX, 64 μg/ml for LE, and 16 μg/ml for GA. These other mutants showed a double substitution in GyrA: Ala118 to Ser and Thr82 to Ala, corresponding to positions 119 and 83 in *E. coli*, respectively. Both substitutions have already been described as single amino acid changes in *Salmonella enterica* and *Flavobacterium psychrophilum*, respectively (13, 21, 24).

C. difficile A422 mutants were characterized by MICs of 32 μg/ml for MX, 64 μg/ml for LE, and ≥32 μg/ml for GA. These mutants showed the amino acid substitution Asp to Asn at position 81 of GyrA, equivalent to position 82 of *E. coli*. This substitution has recently been observed in *C. difficile* strains isolated in China with a phenotype identical to that of the mutants obtained in this study (22).

In GyrB of *E. coli*, substitutions conferring resistance to fluoroquinolones have been described at positions 426 and 447 (19, 36). *C. difficile* 630 mutants had MICs of 16 μg/ml for MX, 64 μg/ml for LE, and ≥32 μg/ml for GA and showed the amino acid change Asp426 to Val in GyrB, already described in toxin A-negative/toxin B-positive *C. difficile* epidemic strains of recent isolation (11). The mutants derived from the nontoxigenic strain BI2 showed one substitution, Arg447 to Lys, in GyrB at the first step of selection and acquired a second amino acid substitution, Asp426 to Asn, at the second step. The first substitution has previously been observed as a single amino acid change in two other nontoxigenic *C. difficile* clinical isolates resistant to fluoroquinolones (38). Mutants showing the first amino acid substitution had MICs of 16 μg/ml for MX, 8 μg/ml for LE, and 1.5 μg/ml for GA, whereas the mutants with a double amino acid change showed increased MICs for MX, LE, and GA: 128, 64, and 6 μg/ml, respectively.

In the majority of the strains, fluoroquinolone resistance is caused by alterations in the *gyrA* and/or *parC* gene, whereas few substitutions in *gyrB* have been described in the bacteria analyzed so far (19, 35, 36). To our knowledge, this is the first time that two substitutions were observed simultaneously in GyrB in a bacterium resistant to fluoroquinolones. The results indicate that double substitutions can be observed in *C. difficile* GyrB by exposing mutants with a single substitution to higher concentrations of fluoroquinolones. Double substitution in *gyrB* seems to be associated with an increase in fluoroquinolone resistance, as observed in bacteria showing multiple substitutions in GyrA (36).

TABLE 3. Phenotypic and genotypic characteristics of *C. difficile* mutants obtained after selection by LE

Parent strain	Mutants selected	Step of selection (μg/ml LE)	MIC (μg/ml)			Substitution(s) in:	
			Agar dilution method for:		Etest for GA		
			LE	MX		GyrA	GyrB
C253	C253 A, B, C	I (4)	4	1	0.5		
	C253 D, E, F	II (8) III (16)	8	2	1		
CD5	CD5 A, B, C	I (4)	4	1	0.5		Ser416 to Ala
	CD5 D, E, F	II (8) III (16)	8	2	2		Ser416 to Ala
A422	A422 A, B, C	I (4)	4	1	1		
	A422 D, E, F	II (8)	8	2	2		Leu451 to Phe
	A422 G, H, I	III (16)	256	64	≥32	Thr82 to Ile	Leu451 to Phe
630	630 A, B, C	I (4)	4	1	0.75		
	630 D, E, F	II (8)	32	8	3	Ala118 to Val	
BI2	BI2 A, B, C	I (4)	4	1	2		
	BI2 D, E, F	II (8)	64	16	≥32		Glu466 to Lys

The colonies derived from C253, CD5, A422, and 630 and isolated at steps I and II showed decreased susceptibility to the fluoroquinolones tested, with MICs between 2 and 4 μg/ml for MX, 0.75 and 2 μg/ml for GA, and 2 and 4 μg/ml for LE (Table 2). The analysis of the QRDR sequences of both *gyrA* and *gyrB* demonstrated the absence of Gyr substitutions in these colonies.

Characterization of *C. difficile* mutants selected by LE. Stable mutants showing Gyr substitutions were obtained from three parent strains, A422, 630, and BI2. The frequency of mutation was 1.4×10^{-6} for A422, 1.0×10^{-6} for 630, and 2.4×10^{-5} for BI2.

In total, 33 colonies were isolated after selection by LE and their phenotypic and genotypic characteristics are shown in Table 3. In general, the isolates were characterized by MICs ranging from 4 to 256 μg/ml for LE, from 1 to 64 μg/ml for MX, and 0.5 to ≥32 μg/ml for GA. Increased MICs and resistance were reached at the second step of selection (8 μg/ml) for 630 and BI2 mutants and at the third step (16 μg/ml) for A422 mutants, after the acquisition of a substitution in GyrA or GyrB.

Mutants obtained at step II from *C. difficile* A422 showed MICs of 8 μg/ml for LE, 2 μg/ml for MX, and 2 μg/ml for GA and were characterized by the substitution Leu to Phe at position 451 of GyrB. The role of this substitution is uncertain because this amino acid change has not been detected in GyrB in resistant *C. difficile* or other bacteria, and A422 mutants, showing a low level of resistance to LE, remained susceptible to MX and GA. However, A422 mutants become highly resistant to all the fluoroquinolones tested (MICs of 256 μg/ml for LE, 64 μg/ml for MX, and ≥32 μg/ml for GA) after the acquisition of a second substitution, Thr82 to Ile in GyrA, at the third step of selection. As mentioned above, this substitution characterized the epidemic clone III/027/NAP1 and is present in the majority of toxigenic *C. difficile* clinical isolates resistant to fluoroquinolones (11, 38).

C. difficile 630 mutants had MICs of 32 μg/ml for LE, 8 μg/ml for MX, and 3 μg/ml for GA and showed the substitution Ala to Val at position 118 of GyrA. This position, corresponding to position 119 in *E. coli*, has a role in fluoroquinolone resistance as already reported above (13, 21). Furthermore, a change from Ala118 to Thr was also observed in a clinical isolate of *C. difficile* resistant to ciprofloxacin and MX (10).

At the second step of selection, mutants resistant to fluoroquinolones from the parent strain BI2 were obtained. These mutants had MICs of 64 μg/ml for LE, 16 μg/ml for MX, and ≥32 μg/ml for GA and showed the amino acid change Glu466 to Lys in GyrB. Recently, this substitution was observed in mutants from a *C. difficile* III/027/NAP1 isolate, already resistant to fluoroquinolones for the presence of the substitution Thr82 to Ile in GyrA, after fluoroquinolone exposure in a human gut model (37). The mutants presenting this second amino acid change showed an increase in the MICs for MX compared to those for the parent strain.

The colonies from CD5 and C253 isolated at step II showed MICs of 8 μg/ml for LE and were susceptible to MX and GA. Sequence analysis showed no substitutions in the QRDR of both *gyrA* and *gyrB* of these colonies.

Presence of efflux systems sensitive to reserpine and CCCP. *C. difficile* parent strains and all the colonies with reduced susceptibility or resistance to MX and LE were tested for efflux systems sensitive to reserpine and CCCP. The presence of an active efflux system seems unlikely in these strains since no MIC variations were observed in the presence of these inhibitors (data not shown).

DISCUSSION

Epidemic fluoroquinolone-resistant *C. difficile* is now a worldwide problem that requires a better understanding of the mechanisms by which this resistance may develop and spread in the *C. difficile* population. Recent studies indicate that the rate of resistance to MX in *C. difficile* has increased dramatically compared to the past and that strains resistant to this antibiotic are also resistant to other fluoroquinolones, in particular to LE, with high levels of MICs (22, 38). Furthermore, the evidence for an association between fluoroquinolone use and CDI, especially CDI due to the hypervirulent *C. difficile* clone III/027/NAP1 which is fluoroquinolone resistant, is be-

coming stronger. For these reasons, we investigated the in vitro capability of MX and LE to select resistant mutants from five susceptible strains, including a *C. difficile* III/027/NAP1 isolate.

Our results showed that both MX and LE were able in vitro to select *C. difficile* mutants showing amino acid substitutions in GyrA and/or GyrB and that were highly resistant to at least two of the fluoroquinolones tested. The majority of the substitutions observed in this study has already been described for clinical isolates or has occurred at positions known to be involved in fluoroquinolone resistance. Interestingly, mutants from *C. difficile* A422, a III/027/NAP1 isolate, showed the amino acid substitution Thr82 to Ile in GyrA, at the second step of selection by LE. Differently, this substitution, characterizing not only the circulating hypervirulent clone III/027/NAP1 but the majority of *C. difficile* strains resistant to fluoroquinolones (11, 28), was not observed in mutants obtained after selection by MX. The in vitro incapacity of MX to select mutants showing the substitution Thr82 to Ile is also supported by a previous study by Ackermann et al. that did not observe any variations at position 82 in the GyrA sequence of four resistant *C. difficile* ATCC 43255 mutants obtained after selection by this antibiotic (1). In that study, the authors used a couple of primers amplifying only 217 bp of the *gyrA* QRDR and did not sequence *gyrB*, so we can speculate that those mutants could present a substitution in a nucleotide region not analyzed. On the other hand, in the present study we observed that MX is able to select mutants showing the substitution Asp to Val at position 426 of GyrB, an amino acid change that characterizes toxin A-negative/toxin B-positive *C. difficile* strains previously recognized as the cause of severe outbreaks (12). The limited number of parent strains analyzed and the in vitro experimental conditions do not allow us to draw a decisive conclusion from these data, but we can hypothesize that the long and extensive use of older fluoroquinolones, in particular LE, in hospital settings could have contributed to the insurgence of resistance in the *C. difficile* population, selecting amino acid substitutions conferring resistance also to the new compounds.

An intriguing observation is the reduced susceptibility to fluoroquinolones in the absence of any amino acid substitution in GyrA or GyrB showed by some colonies isolated after the first and second steps of selection by both MX and LE. Active efflux is a mechanism of low-level fluoroquinolone resistance recognized in different bacteria (19, 20, 36). For this reason, the efflux inhibitors reserpine and CCCP were used in this study to investigate if the strains analyzed showed evidence of an efflux system sensitive to these inhibitors. Since reserpine and CCCP did not have any effect upon MIC levels of the *C. difficile* strains used in this study, the following hypotheses may be considered to explain these data: (i) the presence of other mechanisms of resistance (i.e., efflux pump proteins insensitive to the inhibitors tested), (ii) substitutions occurring outside the QRDR able to interfere with the bond between antibiotic and target, and (iii) the presence of an unknown mechanism of resistance able to affect the MICs. Decreased susceptibility and a low level of resistance may contribute to the emergence and spread of highly resistant bacteria, since surviving cells have the potential to mutate into cells with a higher and clinically more important level of resistance to fluoroquinolones. Studies performed with MX have demonstrated that in subjects receiving 400 mg MX once daily for 7 days, the mean fecal concentration of MX is between 16.3 mg/kg of body weight on the second day and 65.7 mg/kg on the seventh day. MICs for the fecal microorganisms are usually below these concentrations (40). Nevertheless, the main part of the aerobic gram-positive and the anaerobic flora is not significantly affected in number during the administration period (15). This paradox may in part be explained by the reversible binding of antibiotic to bacteria and fecal material such that the amount of active drug in vivo is considerably reduced (14, 15). It may be hypothesized that during the first stages of antibiotic treatment, a subpopulation of *C. difficile* with reduced susceptibility to fluoroquinolones may be able to survive, replicate, produce toxin, and acquire substitutions conferring resistance to higher concentrations of drug, while the antimicrobial level in the intestine is still inhibitory to the remaining population. This phenomenon could be important in determining further increases in fluoroquinolone resistance among *C. difficile* populations and should be considered in controlling resistance.

These in vitro data document the ease, rapidity, and high frequency of selection for various GyrA and GyrB drug-resistant mutants by MX and LE exposure in previously susceptible strains. Also, the presence of identical Gyr changes in both clinical and in vitro-derived fluoroquinolone-resistant *C. difficile* mutants strengthens the relevance of the in vitro observations. While this report examines MX and LE exposure, it is generally observed that the development of fluoroquinolone resistance is a class effect, such that all fluoroquinolones could have the potential for similar selections of resistance. We conclude that the rapid and high frequency of selection for GyrA and GyrB drug-resistant *C. difficile* strains following fluoroquinolone exposure is a clarion call to reduce the current widespread overuse of MX and LE, especially in light of the rising rates of *C. difficile* infection in many countries.

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