

Resistance to Moxifloxacin in Toxigenic *Clostridium difficile* Isolates Is Associated with Mutations in *gyrA*

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Clostridium difficile is the etiological agent of antibiotic-associated colitis and the most common cause of hospital-acquired infectious diarrhea. Fluoroquinolones such as ciprofloxacin are associated with lower risks of *C. difficile*-associated diarrhea. In this study, we have analyzed 72 *C. difficile* isolates obtained from patients with different clinical courses of disease, such as toxic megacolon and relapses; the hospital environment; public places; and horses. They were investigated for their susceptibilities to moxifloxacin (MXF), metronidazole (MEO), and vancomycin (VAN). Mutants highly resistant to fluoroquinolones were selected in vitro by stepwise exposure to increasing concentrations of MXF. The resulting mutants were analyzed for the presence of mutations in the quinolone resistance-determining regions of DNA gyrase (*gyrA*), the production of toxins A and B, and the epidemiological relationship of these isolates. These factors were also investigated using PCR-based methods. All strains tested were susceptible to MEO and VAN. Twenty-six percent of the clinical isolates (19 of 72) were highly resistant to MXF (MIC \geq 16 μ g/ml). Fourteen of these 19 strains contained nucleotide changes resulting in amino acid substitutions at position 83 in the *gyrA* protein. Resistant strains selected in vitro did not contain mutations at that position. These findings indicate that resistance to MXF in a majority of cases may be due to amino acid substitution in the *gyrA* gene.

Clostridium difficile is the major cause of hospital-acquired infectious diarrhea (29). Strains of *C. difficile* produce two toxins, an enterotoxin (toxin A) and a cytotoxin (toxin B), which are the largest bacterial toxins known (molecular masses of 308 and 207 kDa, respectively) (2). Most toxigenic strains release both toxins; however, strains deficient in the production of either toxin A or toxin B have been described (6, 33). Antibiotic exposure and changes of environmental conditions affect toxin production (17, 21, 24, 26).

A prolonged course of antibiotic treatment or the use of two or more antibiotics in combination increases the risk of *C. difficile*-associated diarrhea (CDAD) (14). Vancomycin (VAN) and metronidazole (MEO) are first-line antibiotics for the treatment of CDAD. However, about 15 to 20% of patients relapse after discontinuation of antimicrobial treatment (35). Several studies identified the oral use of VAN in the treatment of CDAD as a risk factor for colonization with VAN-resistant enterococci (13, 27). Therefore, there is still a need for development of new treatments.

Fluoroquinolones are potent, synthetic, antimicrobial agents that are increasingly used in the treatment of human infections. The new-generation quinolones, like gatifloxacin (GAT), trovafloxacin (TVA), and moxifloxacin (MXF), are characterized by improved activity against gram-positive cocci as well as against some gram-positive and -negative anaerobic bacteria

(22). MXF, approved by the Food and Drug Administration in 1999, is available only as an oral preparation and shows a high level of activity against many anaerobes (1, 10, 37). There are no data available concerning the genetic basis for quinolone resistance in *C. difficile* strains. Moreover, a possible relationship between antibiotic treatment and toxin production is obscure.

Since the NCCLS has not yet approved breakpoints for MXF and anaerobes, in this study strains for which MICs were \leq 2 μ g/ml were interpreted as susceptible and those for which MICs were \geq 8 μ g/ml were interpreted as resistant according to breakpoints published for TVA and anaerobic bacteria (23).

Resistance to fluoroquinolones is mediated either by target mutations affecting the genes *gyrA* and *gyrB* for the DNA gyrase subunits A and B as well as *parC* and *parE* for the respective subunits A and B of DNA topoisomerase IV or by mutations resulting in the reduction of the intracellular accumulation of the drug. Mutations in the highly conserved quinolone-resistance-determining regions (QRDR) occur in a different order in gram-positive and gram-negative organisms: DNA gyrase seems to be the primary target of most quinolones in gram-negative bacteria and mycobacteria (19, 30). However, DNA topoisomerase IV appears to be the primary target in gram-positive bacteria (11). Additionally, depending on the fluoroquinolone, *parC* and *gyrA* may be interchangeable targets leading to different levels of resistance (25, 34).

Few data exist about the epidemiology and the mechanisms of resistance to fluoroquinolones in anaerobes, probably due to the low activity of ciprofloxacin (CIP) in contrast to that of newer derivatives, like MXF.

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TABLE 1. *C. difficile* strains resistant to MXF investigated in this study and their sources

Isolate(s)	Source	Reference
1542, 2897, 2923, 2518	Patients with CDAD, toxigenic strains, Leipzig, Germany	This study
1000, 1001, 1002	Toxic megacolon, UCDMC, ^a Sacramento, Calif.	7
1009	Relapse patients, toxigenic strains, UCDMC	This study
1111/1, 1111/2	Relapse patient, toxigenic strains, Mercy Hospital, Sacramento, Calif.	This study
E 213, E 214, E 239, E 287, E 496, E 497, E 498, E 510, E 511	Hospital environment, bathrooms, bedrooms, (all but E 213 and E 214 are toxigenic strains), UCDMC	5
6MX4, 6MX8, 9MX16, 9MX32	In vitro selection	This study

^a UCDMC, University of California-Davis Medical Center.

In this study, 72 *C. difficile* isolates recovered from patients with different clinical courses and from hospital and public environments were investigated for their susceptibility to MXF, MEO, and VAN and for harboring mutations in the QRDR of DNA gyrase (*gyrA*), toxin production, and genotype. The results of this study provide data on the epidemiology of the antimicrobial susceptibility of *C. difficile*.

MATERIALS AND METHODS

Antibiotics. MXF was kindly supplied by Bayer AG, Wuppertal, Germany.

***C. difficile* strains.** The strains investigated in this study were ATCC 43255 and 17857 and strains recovered from patients (*n* = 49), the hospital or public environment (*n* = 12 and *n* = 5, respectively), and horses (*n* = 4) (20). The sources of the MXF-resistant isolates are listed in Table 1. All strains were grown anaerobically on the selective medium cycloserine-cefoxitin-fructose agar (12) at 37°C for 48 h. Isolates were identified as *C. difficile* with the latex agglutination test (Becton Dickinson, Cockeysville, Md.) and the ProDisc test (Remel,

TABLE 2. Nucleotide sequence of PCR primers used to amplify the *gyrA* gene (C 94V, C 121V, C 316R, C 358R, CdgaV, and CdgaR) and 16S rDNA (609V and 699R) of *C. difficile*

Primer	Sequence
C 94V	5'-CGTGCAC(T/G/C/T)CC(A/C/G)GATGT-3'
C 121V	5'-CTTAAGCC(A/G/T)GTTTCAT(A/C)G-3'
C 316R	5'-GAACCAAAGTT(A/T)CC(G/T/A)TG-3'
C 358R	5'-GCTTCTGT(A/G)TA(A/T)C(T/G)CAT-3'
CdgaV	5'-TTTAAAGCCAGTTCATAG-3'
CdgaR	5'-GAACCAAAGTTACCATG-3'
609V	5'-GGATTAGATACCC(C/G/T)(A/G/T)GTA-3'
699R	5'-(A/T/G)GGTTGCGCTCGTT(A/G)C-3'

Norcross, Ga.). PCR was used to identify toxin A and toxin B genes. Selected strains were subjected to 16S ribosomal DNA (rDNA) PCR. Strains were maintained in cooked meat broth (Hardy Diagnostics, Santa Maria, Calif.).

Antimicrobial susceptibility testing. MICs were determined using the broth microdilution technique performed in brucella broth, according to the recommendations of the NCCLS (23), and with Etest (AB BIODISK, Solna, Sweden). Etest was performed by inoculating the surface of prerduced brucella agar plates containing vitamin K1, hemin, and 5% defibrinated sheep red blood cells with a 1 McFarland standard-matched inoculum. The inoculation was done with cotton-tipped swabs that were dipped into the inoculum, pressed against the inside wall of the test tube to remove excess fluid, and streaked three times, with the plate being rotated approximately 90° each time to ensure an even distribution of inoculum. Etest strips were used according to the manufacturer's instructions.

Selection of quinolone-resistant mutants. Selection of quinolone-resistant mutants of ATCC strain 43255 was performed in liquid culture. Bacteria collected from five agar plates (after 48 h of growth) were suspended in 10 ml of brain heart infusion (BHI) broth and were centrifuged for 10 min at 2,700 × *g*. The pellet was resuspended in 2 ml of BHI. Fifty microliters of the suspension was added to 2 ml of BHI containing four different concentrations of MXF (0.5, 1, 2, and 4 µg/ml). After 24 h, cultures which showed visible growth were plated on brucella agar, centrifuged, washed in BHI, and exposed one more time to the same antibiotic concentration. For the next passage, 1-dilution-higher MXF concentrations (1, 2, 4, and 8 µg/ml) were used. That procedure was repeated six and nine times, finally exposing the bacteria to 32 µg of MXF/ml.

DNA extraction. An isolated colony from each strain was transferred with an inoculating loop into a 0.6-ml tube containing 100 µl of sterile water, boiled at 100°C for 10 min, and centrifuged at low speed (3,000 × *g*) to remove cell debris. The DNA-containing supernatant was used for amplification and genotyping reactions.

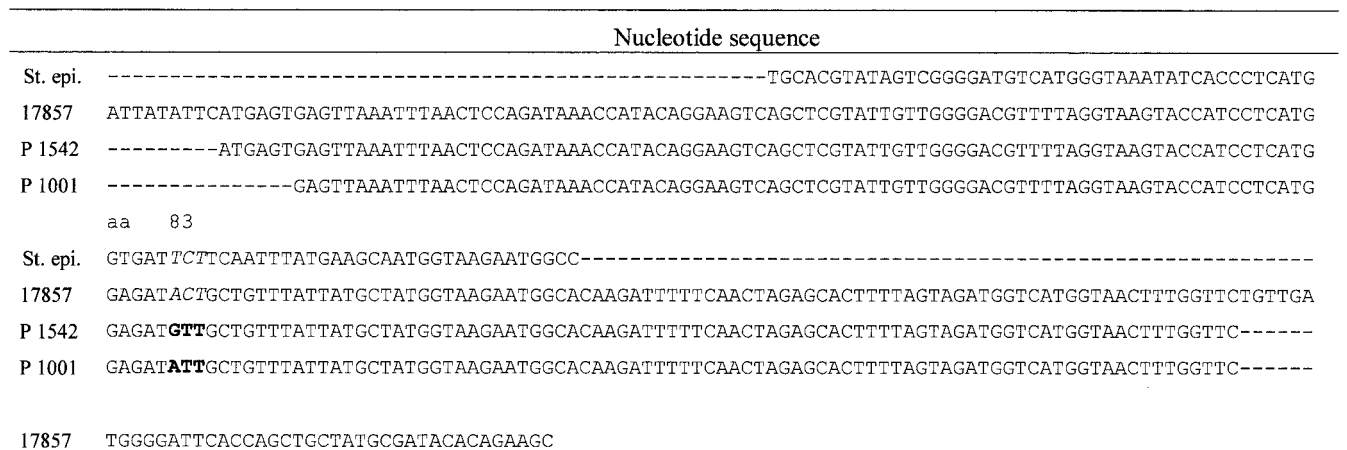


FIG. 1. ORDR nucleotide sequence of the amplified fragment of the *gyrA* gene of *C. difficile* ATCC 17857, 1542 and 1001 and a part of the published sequence for *Staphylococcus epidermidis* (St. epi.). Italics indicate the wild-type sequence at amino acid position 83; boldface indicates nucleotide mutation leading to amino acid substitution; *E. coli* coordinates are given for amino acid sequence.

TABLE 3. Antimicrobial susceptibilities, MIC₅₀ and MIC₉₀, *gyrA* sequence, production of toxins A and B (Tox A/B), and AP-PCR type (AP type) of 19 MXF-resistant *C. difficile* isolates, 4 in vitro-selected strains, and 3 ATCC strains (ATCC 17857 and ATCC 43255, *C. difficile*; ATCC 25285, *Bacteroides fragilis*^{a,b,e})

Strain	MIC (μg/ml)										GyrA aa/nt ^c	Tox A/B	AP type
	Group I	Group II	Group III	Group IV									
	NOR	CIP	GRX	GAT	GMX	TVA	CLX	MXF	MEO	VAN			
ATCC 17857	8	>32	16	2	2	2	0.5	2	0.125	1.5	T/ACT	-/-	NT ^d
ATCC 43255	8	>32	2	1	1	1	0.5	0.5	0.047	1	T/ACT	+/+	NT
ATCC 25285	4	>32	4	0.5	1	1	0.5	0.5	0.5	NT	NT	NT	NT
1542	>64	>32	32	16	32	16	2	16	0.38	1	V/GTT	+/+	2
2897	>64	>32	32	>32	32	32	4	>32	0.125	0.75	I/ATT	+/+	2
2923	>64	>32	32	32	32	32	2	16	0.25	1	T/ACT	+/+	1
2518	>64	32	32	32	8	4	2	>32	0.125	1	T/ACT	+/+	3
1000	>64	>32	32	32	32	32	2	32	0.125	1	I/ATT	+/+	5A,B
1001	>64	>32	32	16	32	32	2	16	0.19	1	I/ATT	+/+	5A,B
1002	>64	>32	32	32	32	32	2	16	0.125	1	I/ATT	+/+	5A,B
1009	>64	>32	32	32	16	32	2	16	0.125	0.75	I/ATT	+/+	5A,B
1111/1	>64	>32	32	32	32	32	2	16	0.125	0.5	IATT	+/+	4
1111/2	>64	>32	32	32	32	32	2	16	1	1.5	IATT	+/+	4
E 213	>64	>32	32	32	16	16	2	16	0.125	0.75	T/ACT	-/-	5A,B
E 214	>64	>32	>32	32	16	32	2	16	0.094	0.75	T/ACT	-/-	6
E 239	>64	16	16	16	16	32	2	>32	0.094	0.25	IATT	+/+	7
E 287	>64	>32	>32	32	32	32	2	32	0.094	1	IATT	+/+	5A,B
E 496	>64	>32	32	32	32	32	2	16	0.5	0.75	IATT	+/+	5A,B
E 497	>64	>32	32	32	32	32	2	16	0.19	1	T/ACT	+/+	5A,B
E 498	>64	>32	32	32	16	32	2	16	0.5	1	IATT	+/+	6
E 510	>64	>32	16	32	16	32	2	16	0.125	0.75	IATT	+/+	5A,B
E 511	>64	>32	32	32	32	32	2	16	0.094	1	IATT	+/+	6
6MX4	>64	>32	>32	8	16	8	2	8	0.094	1.5	T/ACT	+/+	NT
6MX8	>64	>32	>32	8	16	4	2	8	0.064	1.5	T/ACT	+/+	NT
9MX16	>64	>32	32	8	8	4	2	4	0.023	1	T/ACT	+/+	NT
9MX32	>64	>32	32	8	8	4	2	4	0.016	0.75	T/ACT	+/+	NT

^a NOR, norfloxacin; GRX, grepafloxacin; GMX, gemifloxacin; and CLX, clinafloxacin.

^b Groups I to IV are fluoroquinolone groups (22).

^c aa, amino acid; nt, nucleotides. *E. coli* coordinates are given; boldface indicates mutations leading to amino acid substitution.

^d NT, not tested.

^e MIC₅₀s for NOR, CIP, GRX, GAT, GMX, TVA, CLX, MXF, MEO, and VAN are >64, >32, 32, 32, 32, 32, 2, 16, 0.125, and 1 μg/ml, respectively. MIC₉₀s for the same drugs are >64, >32, 32, 32, 32, 32, 2, >32, 0.5, and 1 μg/ml, respectively.

Amplification and sequencing of *gyrA*. A 247-bp fragment of *gyrA* was amplified using degenerated primers developed from consensus regions of *Clostridium acetobutylicum* (C 94V, C 121V, C 316R, and C 358R) (Table 2). The DNA sequence of *C. difficile gyrA* was determined and used to design specific primers (CdgaV and CdgaR) (Table 2 and Fig. 1). Thirty cycles of the following PCR profile were run: 30 s at 95°C, 30 s at 48°C, and 60 s at 72°C. The resulting DNA fragments were purified with Amicon Microcon-PCR Centrifugal Filter Devices (Millipore Corporation, Bedford, Mass.). Complementary strands were sequenced on an ABI310 sequencer (Perkin-Elmer Applied Biosystems, Foster City, Calif.) using either PCR primer.

Primers for 16S rDNA amplification (609V and 699R) (Table 2) were designed using 16S rRNA sequences published by Brosius et al. (3). Sequencing of 16S rDNA identified these strains as *C. difficile*.

Comparison of sequences and design of primers. Sequences were compared using ARB software (O. Strunk and W. Ludwig, <http://www.mikro.biologie.tu-muenchen.de>). This program was primarily designed for phylogenetic analysis of rRNA sequence data but can be used for any other RNA or DNA sequence comparison. Nucleic acid sequences from public databases and those obtained in this study were manually aligned according to the amino acid alignment. The distance-matrix method implemented in the program was used for calculating sequence similarities. In order to amplify *parC* of *C. difficile*, the DNA sequence of the following bacteria was used for developing degenerated primers: *Bacillus subtilis*, *Mycoplasma genitalium*, *Mycoplasma hominis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes*. These are all so-called "low G+C gram-positive bacteria" which are closely related phylogenetically to *C. difficile*.

Amplification of toxin A and B genes. Toxin A and B genes were amplified as described elsewhere (15, 32).

DNA fingerprinting. Arbitrarily primed PCR (AP-PCR) was performed with the 19-mer oligonucleotide T-7 used as the primer. The DNA banding patterns

were compared by running PCR products on the same gel as described previously (9, 29, 31).

RESULTS

Antimicrobial susceptibility. All 72 strains analyzed were susceptible to MEO. The MIC at which 50% of the isolates tested were inhibited (MIC₅₀) and MIC₉₀ were 0.125 and 0.5 μg/ml, respectively. They were also susceptible to VAN; the VAN MIC₅₀ and MIC₉₀ were 1 μg/ml. The MXF MIC₅₀ and MIC₉₀ were 1 and ≥ 32 μg/ml. For 19 (26%) of the 72 *C. difficile* isolates studied, MXF MICs were ≥ 16 μg/ml as determined by Etest. Two MXF-resistant isolates were recovered from one patient (1111/1 and 1111/2). They showed differences in growth phenotype and in antimicrobial susceptibility for MEO and VAN by 3 or 4 dilutions.

Nine of twelve isolates from the hospital environment were resistant to MXF, while all strains isolated from the non-hospital environment were susceptible to MXF (five of five). The three isolates from patients with toxic megacolon were highly MXF resistant (1000, 1001, and 1002).

The 19 strains resistant to MXF were tested with the broth microdilution method against 12 other quinolones (one agent each represented groups I to III; all tested group IV fluoroquinolones are shown in Table 3). The MICs of MXF deter-

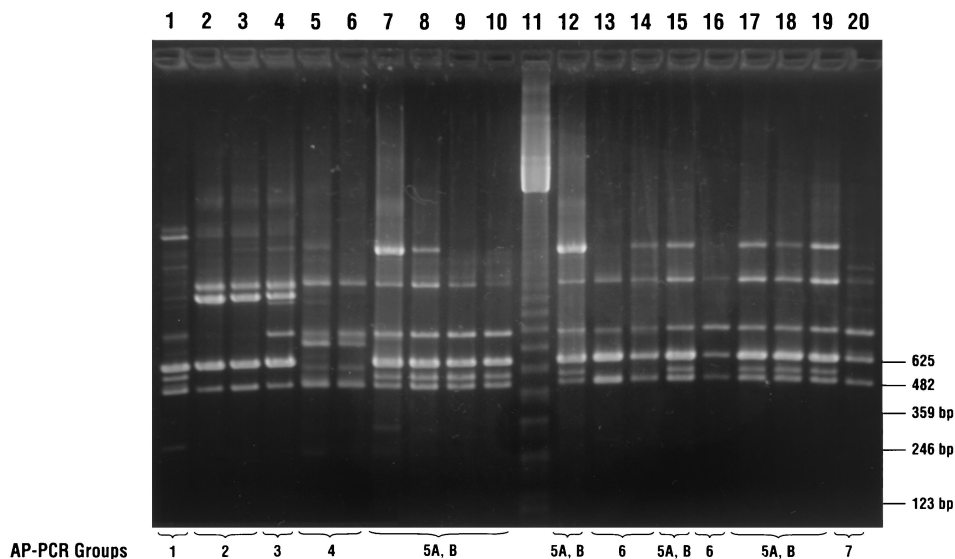


FIG. 2. Genotyping of *C. difficile* isolates by AP-PCR with a T-7 primer. Lane 11, 123 bp-ladder; lanes 1 to 10 and 12 to 20, AP-PCR banding patterns of MXF-resistant isolates. Lanes and isolates: 1, 2932; 2, 1542; 3, 2897; 4, 2518; 5, 1111/1; 6, 1111/2; 7, 1009; 8, 1002; 9, 1001; 10, 1000; 12, E 214; 13, E 213; 14, E 511; 15, E 510; 16, E 498; 17, E 497; 18, E 496; 19, E 287; and 20, E 239. AP-PCR types are indicated by numbers beneath the lanes.

mined by the broth microdilution method were in general 1 or 2 dilutions lower than those determined by Etest (MXF MIC₅₀ and MIC₉₀, 16 and ≥ 32 $\mu\text{g/ml}$, respectively), which is within acceptable limits of error for the test. Except for clinafloxacin (MIC₅₀ and MIC₉₀, both 2 $\mu\text{g/ml}$), other quinolones did not show activities against the tested isolates (MIC₅₀ and MIC₉₀ in micrograms per milliliter): norfloxacin (both >64), pefloxacin (both >32), CIP (both ≥ 32), fleroxacin (both >32), ofloxacin (both ≥ 32), enoxacin (both 32), grepafloxacin (both 32), sparfloxacin (both >32), GAT (both 32), gemifloxacin (both 32), and TVA (both 32).

ATCC strain 43255 (for which the MXF MIC was 0.5 $\mu\text{g/ml}$) was exposed six and nine times to increasing concentrations of MXF (starting with 0.5 $\mu\text{g/ml}$ and going to 32 $\mu\text{g/ml}$). That resulted in MICs increasing from 8 to >32 $\mu\text{g/ml}$ as determined by Etest (isolates 6MX4, 6MX8, 9MX16, and 9MX32) (Table 3). Once again for these four strains, MICs were 1 or 2 dilutions higher when evaluated with Etest than with the microdilution broth procedure.

GyrA sequence. In *Escherichia coli* and in the closest related *Clostridium* species with a known *gyrA* amino acid sequence, *Clostridium acetobutylicum*, the amino acid serine was found at position 83 (*E. coli* coordinates). The wild-type and quinolone-susceptible strains of *C. difficile* carry the amino acid threonine at position 83.

Fourteen of the 19 MXF-resistant strains contained a mutation at codon 83 (*E. coli* coordinates) in the *gyrA* gene resulting in an amino acid exchange. Thirteen isolates showed the same nucleotide transition (ACT \rightarrow ATT) resulting in a threonine \rightarrow isoleucine change. In one isolate (1542), two nucleotide changes (ACT \rightarrow GTT) resulted in a replacement of threonine by valine (Table 3; Fig. 1). All MXF-susceptible strains had the same sequence found in the two MXF-susceptible *C. difficile* ATCC strains. All four in vitro-selected MXF-resistant strains retained the wild-type sequence at position 83 in the *gyrA* protein.

Toxin production. Forty-two (58%) *C. difficile* isolates produced toxins A and B. Toxin A and B gene sequences were not detected in 30 isolates. Seventeen (89%) of the 19 MXF-resistant strains were toxigenic. The strains recovered from the public environment produced no toxin; however, 9 (75%) of 12 hospital environment strains produced toxins A and B.

Genotyping. Of the 19 MXF-resistant strains, seven different groups, including two subgroups, were identified using the T-7 primer and the Dice similarity coefficient (Fig. 2) (9, 29, 31). No correlation between genotype and antimicrobial susceptibility was detected, as some resistant and susceptible strains had the same genotype.

DISCUSSION

By using degenerated primers developed from conserved QRDR of the *gyrA* gene of *C. acetobutylicum*, we were able to amplify and sequence the corresponding region of the *gyrA* gene from *C. difficile*. The deduced amino acid sequence shows high homology with other *gyrA*-like proteins, such as 83% identity with that of *B. subtilis* (NCBI accession no. Z99104), 81% identity with that of *S. pneumoniae* (accession no. AB010387), 77% identity with that of *S. aureus* (accession no. AF044066), and 82% identity with that of *C. acetobutylicum* (accession no. U35453).

Ser-83 in the QRDR of *E. coli* seems to play a key role in resistance to fluoroquinolones (4). This study constitutes the first genetic characterization of fluoroquinolone resistance in *C. difficile*. Since we did not detect base changes in *gyrA* in 5 of the 19 MXF-resistant strains or in the in vitro-selected strains, resistance in those strains might be due to mutation(s) in topoisomerase IV, the other known target of fluoroquinolones. Additionally, increased drug efflux might be involved in the expression of fluoroquinolone resistance in the mutants. Whether the low level of resistance found in the in vitro-selected mutants is due to the limited drug exposure (consider-

ering that clinical isolates may be more consistently exposed to drugs during treatment) is not known and needs further investigation. According to various studies on quinolone resistance, high-level resistance develops in multiple steps involving several targets and pathways of the antibiotic (11, 16, 36). Another possibility is that the in vitro-selected isolates consist of a mixed population (heteroresistance). Due to the finding that the *parC* gene plays a role as the primary target in gram-positive organisms, further studies will focus on that gene.

Initial attempts to amplify the *parC* gene using degenerated primers developed from a consensus sequence from published *parC* sequences of different gram-positive organisms were unsuccessful. The finding that the only PCR fragments obtainable contained the *gyrA* sequences suggests a very high homology in the nucleotide sequences of the two genes used for the primer design.

The high frequency of MXF resistance among *C. difficile* isolates (26%) is in contrast to the findings of other studies that reported a prevalence of MXF resistance between 0 and 14% (10, 18, 37). The quinolones of groups I, II, and III showed no activity against the MXF-resistant strains. Only clinafloxacin of group IV was active against the strains tested (22). The sources of the strains investigated in this study may be one explanation for the high rate of resistance and suggest a relationship between antibiotic exposure and antibiotic susceptibility. Almost all clinical isolates were recovered from patients with recurrent CDAD or toxic megacolon (Table 1).

The persistence of environmental strains in hospitals is known to be correlated with endemic and nosocomial CDAD (5, 8, 28). Our discovery of a high number of resistant isolates in the hospital environment and the finding that these hospital isolates contain toxin genes confirm the important role of these strains as a possible source for exogenous infections.

The resistance of *C. difficile* strains to newer quinolones raises a number of questions regarding the origin and the development of resistance. Since MXF has been approved for use for only about a year in the United States and for a few months in Europe, it is unlikely to be involved in the selection of resistant mutants. Therefore, the role that the wide clinical use of quinolones, such as CIP, OFX, and levofloxacin, might have played in the development of this resistance should be investigated further. The finding of MXF-resistant isolates in the hospital environment but not in public places (parks and public bathrooms) indicates the need for further studies.

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