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gyrA and *gyrB* Mutations Are Implicated in Cross-Resistance to Ciprofloxacin and Moxifloxacin in *Clostridium difficile*

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A total of 198 nonrepetitive clinical strains of *Clostridium difficile* isolated from different French hospitals in 1991 ($n = 100$) and 1997 ($n = 98$) were screened for decreased susceptibility to fluoroquinolones by plating onto Wilkins-Chalgren agar containing 16 μg of ciprofloxacin per ml. The frequency of decreased susceptibility was 7% (14 of 198) and was identical for the years 1991 and 1997. Serogroups C, H, D, A9, and K accounted for five, four, two, one, and one of the resistant strains, respectively, one strain being nontypeable. Arbitrarily primed PCR typing showed that all resistant strains had unique patterns except two serotype C strains, which could not be clearly distinguished. All isolates with decreased susceptibility carried a mutation either in *gyrA* (eight mutations, amino acid changes Asp71→Val in one, Thr82→Ile in six, and Ala118→Thr in one) or in *gyrB* (six mutations, amino acid changes Asp426→Asn in five and Arg447→Leu in one). These changes are similar to those already described in other species except for Asp71→Val, which is novel, and Ala118→Thr, which is exceptional. Attempts to detect the topoisomerase IV *parC* gene by PCR amplification with universal *parC* primers or DNA-DNA hybridization under low-stringency conditions were unsuccessful. The susceptibilities of all resistant strains to ciprofloxacin and ethidium bromide were not affected by the addition of reserpine at 20 μg/ml. In conclusion, decreased susceptibility to fluoroquinolones in *C. difficile* is rare in France and is associated with the occurrence of a *gyrA* or *gyrB* mutation.

Vancomycin and metronidazole are effective antibiotics for the treatment of *Clostridium difficile*-associated diarrhea (19, 24). However, at least 15% of patients relapse after discontinuation of treatment for poorly understood reasons (23, 24). Other treatments may be more effective in preventing relapses.

The in vitro activities against *C. difficile* of older fluoroquinolones like ciprofloxacin and ofloxacin are poor, but those of some new compounds like moxifloxacin are markedly increased (1, 3, 17, 25). Thus, these drugs could become therapeutic alternatives for the treatment of *C. difficile*-associated diarrhea. Decreased susceptibility to fluoroquinolones in *C. difficile* has already been described (2, 3, 13, 26), but the data reported are controversial and the genetic mechanisms involved are poorly understood.

In the present work, 198 French clinical strains of *C. difficile* were screened for decreased susceptibility to fluoroquinolones, and the genetic basis of this phenomenon was investigated.

MATERIALS AND METHODS

Bacterial strains. Reference strain ATCC 9689 and 198 nonrepetitive clinical isolates obtained in 1991 ($n = 100$) and 1997 ($n = 98$) from 36 French hospitals (7) were studied. Serotyping of the strains had been performed in a previous study (7) by the method of Delmée et al. (12). Serogroups A, C, D, F, G, H, and K accounted for 9, 22, 8, 1, 4, 19, and 14% of the strains, respectively, and 23% of the strains were untypeable. Strains were cultured in an anaerobic atmosphere at 37°C for 48 h on TCCA medium (brain heart agar supplemented with 5% defibrinated horse blood, 0.1% taurocholate, 250 μg of cycloserine per ml, and 10 μg of cefoxitin per ml).

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Drug susceptibility testing. We first screened *C. difficile* strains for decreased susceptibility to fluoroquinolones. The strains were grown at 37°C for 24 h in prereduced Wilkins-Chalgren broth (Oxoid, Dardilly, France), 10^5 CFU were applied on prereduced Wilkins-Chalgren agar (Oxoid) containing 16 μg of ciprofloxacin per ml, and the plates were incubated for 48 h at 37°C in an anaerobic atmosphere. The inoculum, medium, and incubation time used were those recommended for anaerobes by the Comité de l'Antibiogramme of the Société Française de Microbiologie (11).

For the strains with decreased susceptibility as well as for reference strain ATCC 9689, MICs of ciprofloxacin, moxifloxacin, and certain drugs (ethidium bromide, acridine orange, safranin, and acriflavin) were determined by the agar dilution method with the same culture conditions and inoculum that were employed for the screening. MICs of ciprofloxacin and ethidium bromide were also determined in the presence of 20 μg of reserpine (ICN Pharmaceuticals, Orsay, France) per ml.

The susceptibilities of these strains to other antibiotics (chloramphenicol, erythromycin, tetracycline, and rifampin) had been determined in a previous study with the ATB ANA susceptibility test (bioMérieux, Marcy l'Etoile, France) (7). Breakpoints used were those recommended by the Comité de l'Antibiogramme of the Société Française de Microbiologie (11).

DNA amplification by PCR and sequencing. For amplification of the quinolone resistance-determining regions of the *gyrA* and *gyrB* genes, oligonucleotide primers were designed from the sequence of the genome of *C. difficile* strain 630 (http://www.sanger.ac.uk/Projects/C_difficile/blast_server.shtml). Primers 5′-AATGAGTGTTATAGCTGGACG-3′ and 5′-TCTTTTAACGACTCATCAAAGTT-3′ were used to amplify a 390-bp fragment of *gyrA* from nucleotide positions 71 to 460. Primers 5′-AGTTGATGAACTGGGGTCTT-3′ and 5′-TCAAAATCTTCTCCAATACCA-3′ were used to amplify a 390-bp fragment of *gyrB* from nucleotide positions 1059 to 1448.

The sequences of both strands were determined by the dideoxy chain termination method with the primers used for PCR and the automatic DNA sequencer ABI Prism 373A (Applied Biosystems, Courtaboeuf, France).

Strain genotyping by AP-PCR. Chromosomal DNA was extracted as described above. Arbitrarily primed PCR (AP-PCR) was performed with primer AP3 (5′-TCACGATGCA-3′) or AP4 (5′-TCACGCTGCA-3′), as described previously (9).

DNA-DNA hybridization. Genomic DNAs from clinical isolate *C. difficile* 698 and reference strain *Escherichia coli* JM109 were obtained by phenol-chloroform

extraction and digested with restriction enzymes *Hin*dIII and *Eco*RI (New England Biolabs, Saint-Quentin en Yvelines, France), respectively. Digested DNAs were transferred after electrophoresis to a nylon membrane and hybridized with [^{32}P]dCTP-labeled PCR-amplified DNA fragments used as probes: a 232-bp fragment of *gyrA* from *E. coli* (4), a 343-bp fragment of *parC* from *E. coli* (4), and the above-described 390-bp fragment of *gyrA* from *C. difficile*.

RESULTS AND DISCUSSION

Decreased susceptibility to fluoroquinolones: frequency and typing of the strains. The frequency of decreased susceptibility to fluoroquinolones, as defined by an MIC of ciprofloxacin higher than 16 μg/ml, was 7% (14 of 198) and was identical for the years 1991 and 1997. The cutoff value of 16 μg/ml was chosen because the MIC that inhibited 50% of strains (MIC_{50}) of ciprofloxacin for *C. difficile* is 8 to 16 μg/ml (3, 24, 25). All 14 strains also showed decreased susceptibility to moxifloxacin (Table 1). Thus, decreased susceptibility to fluoroquinolones in *C. difficile* remains relatively rare in our country (7%), and our results are in agreement with data from Sweden, the Netherlands, and the United Kingdom (13, 17, 26).

However, two studies have reported contradictory results. In Germany (2), 26% (19 of 72) decreased susceptibility to moxifloxacin (MIC > 8 μg/ml) was found in isolates recovered from patients or from the hospital or public environments, but some of the strains were genotypically related. In Spain (3), 30% (34 of 113) of the clinical isolates collected from one hospital had decreased susceptibility to ciprofloxacin (MIC > 16 μg/ml). However, no typing of the isolates was performed, and the isolates were collected over a short period (6 months) and could thus correspond to only a few clones.

In our work, 9 of the 14 strains with decreased susceptibility belonged to two serotypes, C (five strains) and H (four strains) (Table 1). However, these two serotypes were also the most frequently observed among the 198 study strains. The remaining five strains belonged to serotypes D (two) and A9 and K (one each), the last being untypeable. All 14 strains could be clearly distinguished by AP-PCR except for two serotype C strains, 580 and 602 (data not shown). The patterns observed for these strains with primers AP3 and AP4 were different by only two bands and identical, respectively.

Detection of *gyrA* and *gyrB* mutations. The quinolone resistance-determining regions of *gyrA* and *gyrB* were sequenced from nucleotide codons 40 to 145 and 366 to 473, respectively. A mutation in *gyrA* was present in eight strains, leading to the substitutions Thr82→Ile (six strains), Asp71→Val (one strain), and Ala118→Thr (one strain). The remaining six strains carried a mutation in *gyrB* leading to the alterations Asp426→Asn (five strains) and Arg447→Leu (one strain) (Table 1).

Thr82 corresponds to Ser83 in *E. coli*, and that position is known to play a key role in fluoroquinolone resistance in every species (18). In *C. difficile*, 14 of the 19 resistant strains investigated by Ackermann et al. (2) carried a mutation at codon 82 of *gyrA*, leading to the same Thr82→Ile substitution in 13 cases and to Thr82→Val in the last case.

Asp71→Val corresponds to a change at position 72 of GyrA from *E. coli*, which has not yet been associated with fluoroquinolone resistance in any species. It is located in the quinolone resistance-determining region and is very likely to be implicated in resistance, but we do not have direct proof of that. Ala118→Thr corresponds to a change at position 119,

TABLE 1. Phenotypic and genotypic characteristics of the fluoroquinolone-resistant strains of *C. difficile* studied[a]

Strain	Year isolated	Hospital	Toxin production	Serotype	MIC (μg/ml)		Susceptibility				Amino acid substitution (mutation)	
					CIP	MOX	CHL	ERY	TET	RIF	GyrA	GyrB
ATCC9689			−	G	8	2						
253	1991	Bichat	+	C	64	16	R	R	R	R		Asp426→Asn (GAT→AAT)
580	1991	Bichat	+	C	32	8	R	R	R	R		Asp426→Asn (GAT→AAT)
602	1991	Bichat	+	C	128	8	R	R	R	R		Asp426→Asn (GAT→AAT)
714	1991	Bichat	−	D	128	32	S	S	R	S	Thr82→Ile (ACT→ATT)	
1229	1991	Bichat	+	C	128	8	S	R	R	R		Asp426→Asn (GAT→AAT)
728	1991	Pitié	+	A9	128	32	S	S	S	R	Thr82→Ile (ACT→ATT)	
875	1991	Saint-Joseph	+	H	64	8	S	S	S	S	Asp71→Val (GAC→GTC)	
22	1997	Broca	+	K	128	16	S	S	S	S		Asp426→Asn (GAT→AAT)
36	1997	Cochin	+	C	64	16	S	R	R	R	Thr82→Ile (ACT→ATT)	
266	1997	René Muret	+	H	128	32	S	S	S	S	Thr82→Ile (ACT→ATT)	
271	1997	Saint-Antoine	+	H	128	32	S	S	S	S	Thr82→Ile (ACT→ATT)	
302	1997	Saint-Antoine	+	H	128	32	S	S	S	S	Thr82→Ile (ACT→ATT)	
334	1997	Institut Montsouris	+	Nt	32	4	S	S	S	S		Arg447→Leu (CGA→CTA)
358	1997	Broussais	−	D	64	8	S	R	S	S	Ala118→Thr (GCT→ACT)	

[a] MICs of ciprofloxacin (CIP) and moxifloxacin (MOX) were determined by agar dilution. Susceptibility was determined in a previous study with the ATB ANA susceptibility test (7). CHL, chloramphenicol; ERY, erythromycin; TET, tetracycline; RIF, rifampin; Nt, nontypeable.

and other alterations at that position have occasionally been reported in both GyrA and ParC: Ala→Val in GyrA from *Bacteroides fragilis* (5) and *Salmonella enterica* serovar Typhimurium (16); Ala→Pro or Glu in ParC from *Staphylococcus aureus* (20) and *Streptococcus pneumoniae* (8). The role in resistance of this mutation has been demonstrated in *S. aureus* by cloning the mutated *parC* gene into a shuttle vector and introducing it into a wild-type *S. aureus* strain (14).

The GyrB changes detected in *C. difficile* are, compared to those described in *E. coli*, either identical (Asp426→Asn) or located at the same position (Asp447→Lys for *E. coli*) (18). Their frequent occurrence is surprising because in other species *gyrB* mutations have been observed mainly in laboratory mutants (18). However, four of the five *gyrB* mutations that we found were identical and concerned serotype C strains isolated from the same hospital between February and December 1991. As already mentioned, two of these isolates appeared to be genetically related, but the patterns obtained by AP-PCR for the other two isolates were different. However, it cannot be excluded that these four mutants in fact derive from a common ancestor.

Search for other resistance mechanisms. We did not find any gene with homology to known *parC* genes in the genome of *C. difficile* 630. PCR experiments with degenerate primers deduced from conserved regions of the published *parC* genes from other bacterial species (21) were also unsuccessful, as was the case for others (2). Low-stringency DNA-DNA hybridization with probes specific for *gyrA* and *parC* of *E. coli* did allow us to detect the *gyrA* gene of *C. difficile* but not a putative *parC* gene. Taken together, these data suggest that *C. difficile*, like *Mycobacterium tuberculosis* (10), *Helicobacter pylori* (22), *Campylobacter jejuni* (6), and *Treponema pallidum* (15), lacks genes for topoisomerase IV.

We also determined the MICs of certain compounds that can be substrates for efflux pumps. The MICs of acridine orange, acriflavin, ethidium bromide, and safranin for all strains were similar: 256 to 1024, 2 to 8, 8 to 32, and 128 to 256 μg/ml, respectively. Furthermore, the MICs of ciprofloxacin and ethidium bromide for all strains were either unchanged or decreased only twofold after addition of reserpine. Some of our strains were coresistant to chloramphenicol, erythromycin, and tetracyclines (Table 1), but these multiresistant strains have been found, by DNA-DNA hybridization and PCR amplification experiments, to carry resistance genes which are not implicated in efflux, *catD*, *ermB*, and *tetM*, respectively, (N. Hidri, D. Decré, M. Chosson, B. Burghoffer, F. Barbut, and J.-C. Petit, submitted for publication). Thus, the presence of active efflux seems unlikely in the strains studied.

In conclusion, our work shows that decreased susceptibility to both ciprofloxacin and moxifloxacin already exists in French isolates of *C. difficile* but remains relatively rare and is strongly associated with the presence of a *gyrA* or *gyrB* mutation.

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