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In Vitro Development of Resistance to Six and Four Fluoroquinolones in *Mycoplasma pneumoniae* and *Mycoplasma hominis*, Respectively

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Selection of resistant mutants in sequential subcultures with increasing concentrations of six and four different fluoroquinolones was studied for one reference strain each of *Mycoplasma pneumoniae* and *Mycoplasma hominis*, respectively. All fluoroquinolones tested selected for resistance, with alterations affecting the quinolone resistance-determining regions of the four target topoisomerase genes.

Human mycoplasmas, *Mycoplasma pneumoniae* and *Mycoplasma hominis*, are etiological agents of respiratory and genitourinary tract infections, respectively, for which fluoroquinolones offer the potential for empirical treatment. Newer compounds of this class have activity against mycoplasmas that is improved over that of older fluoroquinolones (1). To date, acquired resistance to fluoroquinolones has been reported among human mycoplasma clinical isolates only for the genital species, *M. hominis*, *M. genitalium*, and *Ureaplasma* spp. (3–5). In vivo target alterations located in the quinolone resistance-determining regions (QRDRs) of both DNA gyrase and topoisomerase IV subunits were confirmed by in vitro quinolone resistance studies with *M. hominis* (2, 8).

The purpose of this study was to determine and compare the abilities of six older and newer fluoroquinolones to select for fluoroquinolone-resistant mutants of *M. pneumoniae*. Furthermore, our previous data on fluoroquinolone resistance in *M. hominis* were completed by selecting for mutants of this mycoplasma that were resistant to the newer compounds levofloxacin, moxifloxacin, gemifloxacin, and gatifloxacin.

Growth conditions and antibiotic susceptibility testing of the mycoplasma strains have been previously described (12). MICs of fluoroquinolones were determined in the presence and absence of reserpine (20 μg/ml) as described previously (11). Two selection methods, with either broth or agar medium, were used for *M. pneumoniae* FH (ATCC 15531), while only the agar-based selection was done for *M. hominis* PG21 (ATCC 23114). Broth-selected mutants were obtained by serial transfers of *M. pneumoniae* FH in Hayflick modified broth medium containing subinhibitory concentrations of each fluoroquinolone, as previously described (10). For the first passage, the reference strain *M. pneumoniae* FH was inoculated in Hayflick modified medium with increasing twofold dilutions of each antibiotic. The MIC was determined as the lowest concentration of antimicrobial agent that prevented a color change in the medium at the time when the drug-free growth control first showed a color change (after about 5 days of incubation at 37°C). The culture containing the highest antibiotic concentration with visible growth (subinhibitory concentration) was used to inoculate another antibiotic dilution panel for the following passage. Fifteen passages were performed for each selector antibiotic except for gatifloxacin, and two of the five clones subcultured from passages 5, 7, 10, and 15 were studied. With gatifloxacin, the characterization of passage 2 was added and the selection was conducted up to 10 passages. Subinhibitory concentrations ranged from 0.06 to 16 μg/ml, depending on the selector fluoroquinolone and the selected passage. For the stepwise selection on agar, *M. pneumoniae* FH cells and *M. hominis* PG21 cells were concentrated 100-fold by centrifugation and directly filtered through a 0.45-μm-pore size filter (Millipore) to reach an inoculum titer of approximately 10^9 and 10^{10} color changing units/ml, respectively. Then, selection of fluoroquinolone-resistant mutants was performed as previously described by plating a 100-μl inoculum onto Hayflick modified agar medium containing increasing inhibitory concentrations of the selector antibiotic (2). For each selection experiment, three steps were performed with fluoroquinolone concentrations at 2, 4, 8, and 10 times the MIC for the respective parent strain. The mutation frequency was determined as the number of colonies appearing on the plate with antibiotic divided by the number of colonies in the inoculum.

Amplification of the *gyrA*, *gyrB*, *parC*, and *parE* QRDRs was carried out with 2 μl of a broth culture for the resistant mutants and 1 μM (each) primer, as described elsewhere (2). For *M. pneumoniae*, primer sets were chosen from the complete genome sequence (6) to amplify a 550-bp *gyrA* fragment (nucleotides [nt] 28 to 577), a 297-bp *gyrB* fragment (nt 1258 to 1554), a 588-bp *parC* fragment (nt 22 to 609), and two *parE* fragments of 269 bp (nt 5 to 273) and 300 bp (nt 1219 to 1518). For *M. hominis*, *gyrA* and *parC* fragments were obtained with primer set MHA1 (5′-ATGAGTGTCATAGTTTCTCG-3′) and MH4 (2) and primer set MHC1 (5′-GCCGATATAATG TCTGATAG-3′) and MHC2 (5′-TGTTGCATCAATAACTT CGC-3′), respectively. *gyrB* and *parE* QRDRs were amplified with primers MH6-7 and MH28-29, respectively (2). A 5′ *parE* fragment was also amplified with primers MHE1 (5′-AAATA ATTACGAAGCTAGCG-3′) and MHE2 (5′-ACTCGTGTTT ATTGACAGG-3′). PCR products were directly sequenced by using an ABI PRISM dRhodamine terminator cycle sequencing ready reaction kit (Applied Biosystems).

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TABLE 1. Characteristics of selected fluoroquinolone-resistant mutants of *M. pneumoniae*

Reference strain, selection method, or selected mutant[c]	MIC (μg/ml) of[a]:						Amino acid change(s) in QRDR of[b]:			
	CIP	SPX	LVX	MXF	GEM	GAT	GyrA	GyrB	ParC	ParE
FH	2	0.12	0.5	0.12	0.12	0.12	None	None	None	None
SPX selection										
On agar										
IIPSA	8	1	2	0.5	1	ND[d]	—[e]	—	G81C	—
IIPSC1	8	1	2	0.5	1	ND	—	—	D87Y	—
IIIPSD1	8	2	2	0.5	1	ND	—	—	D87N	—
IIIPSD3	8	1	4	0.5	1	ND	—	—	A83V	—
In broth										
S5A	4	1	1	0.5	0.5	ND	—	—	D87N	—
S7A	16	2	2	1	4	ND	—	E483G	D87N	—
S10A	32	8	16	4	8	ND	—	D443N	D87N	—
LVX selection										
On agar										
IPLA	8	1	2	0.5	0.5	ND	D99N	—	—	—
In broth										
L7A	8	1	4	2	1	ND	D99N	—	—	P449S
L15A	32	16	16	8	4	ND	D99N	—	D87N	P449S
MXF selection										
On agar										
IPMB	2	0.25	1	0.25	0.5	ND	—	E483G	—	—
IIPMB	8	1	2	1	2	ND	—	E483G	D87N	—
In broth										
M5A	16	8	16	2	4	ND	—	D443N	D87N	—
M15A	16	16	16	8	4	ND	—	D443N, R464K	D87N	—
GEM selection										
On agar										
IIIPGB	8	1	2	0.25	1	ND	—	—	D87G	—
In broth										
G5A	8	1	2	1	1	ND	D99N	—	—	—
G7A	16	16	16	2	4	ND	D99N	—	D87G	P449S
CIP selection (in broth only)										
C7A	16	2	2	2	4	ND	—	E483G	G81C	—
C7B	8	2	2	1	1	ND	—	—	G81C	—
GAT selection (in broth only)										
Ga2A	16	2	4	1	8	2	—	E483G	G81C	—

[a] CIP, ciprofloxacin; SPX, sparfloxacin; LVX, levofloxacin; MXF, moxifloxacin; GEM, gemifloxacin; GAT, gatifloxacin.
[b] *M. pneumoniae* positions GyrA 99, GyrB 443, 464, and 483, ParC 81, 83, and 87, and ParE 449 correspond to *E. coli* coordinates GyrA 83, GyrB 426, 447, and 466, ParC 78, 80, and 84, and ParE 439.
[c] *M. pneumoniae* FH is the parental strain. Agar-selected mutants are designated by a prefix corresponding to the selection step (I, II, or III) followed by the initial of the species (P) and of the selector fluoroquinolone (C, ciprofloxacin; S, sparfloxacin; L, levofloxacin; M, moxifloxacin; G, gemifloxacin; Ga, gatifloxacin). Broth-selected mutants are designated by the initial of the selector fluoroquinolone, followed by the passage number. Fifteen passages were performed for each antibiotic. For passages 5, 7, 10, and 15, two of the five clones subcultured were studied except with gatifloxacin, for which passage 2 was also studied. When both clones from one passage were identical, only one clone is represented in this table. For both agar and broth methods, only clones with significantly increased MICs and QRDR mutations are shown. See footnote *a* for three-letter drug abbreviations.
[d] ND, not determined.
[e] —, identical to the reference strain.

We were able to select quinolone-resistant *M. pneumoniae* mutants after serial passages in subinhibitory concentrations of all the six fluoroquinolones used in this study. Two fluoroquinolones, ciprofloxacin and gatifloxacin, selected for *M. pneumoniae* mutants only with the broth method, using subinhibitory antibiotic concentrations. In contrast, resistant *M. hominis* mutants were obtained in the presence of inhibitory concentrations of the four fluoroquinolones studied. For *M. pneumoniae*, mutation frequencies ranged from 1.3×10^{-6} to 2.9×10^{-7} with sparfloxacin, while they ranged from 3×10^{-8} to 7×10^{-9} with levofloxacin, moxifloxacin, and gemifloxacin. For *M. hominis*, overall, mutation rates were lower with moxifloxacin, gemifloxacin, and gatifloxacin (about 10^{-8}) than with levofloxacin (about 10^{-7}). The susceptibility profiles of the mutants according to their *gyrA*, *gyrB*, *parC*, and *parE* QRDR status are shown Table 1 for *M. pneumoniae* and in Table 2 for

TABLE 2. Characteristics of selected fluoroquinolone-resistant mutants of *M. hominis* obtained from agar selection with various drugs

Reference strain, selection method, or selected mutant[c]	MIC (μg/ml) of[a]:					Amino acid change in QRDR of[b]:			
	CIP	LVX	MXF	GEM	GAT	GyrA	GyrB	ParC	ParE
PG21	1	0.25	0.06	0.06	0.12	None	None	None	None
LVX selection									
IHLA	4	4	0.12	0.12	0.5	—[d]	—	—	D426N
IHLB	4	1	0.12	0.12	0.25	—	—	R84H	—
IHLC	4	2	0.12	0.06	0.25	—	—	—	R447K
MXF selection									
IHMA	>128	64	8	8	16	S153L	A453F	S91I	—
IHMI	>128	32	8	8	16	—	V450F	—	E466K
IIHMA	>128	256	32	32	64	S153L	A453F	S91I	D426N
GEM selection									
IHGA	2	0.5	0.12	0.06	0.25	—	—	—	E466K
IHGC	2	0.25	0.12	0.25	0.25	D152N	—	—	—
IIHGB1	32	1	1	4	2	E157K	—	—	E466N
IIHGB9	32	0.5	1	4	2	S153L	—	—	E466K
IIIHB9A	32	0.5	1	8	4	S153L, A163T	—	—	E466K
IIIHB9B	>128	64	16	16	64	S153L	—	S91I	E466K
GAT selection									
IHGaA	4	2	0.06	0.06	0.25	—	—	S91I	—
IHGaC	4	1	0.06	0.06	0.25	—	—	—	E466K
IIHGaB	>128	32	8	8	16	S153L	A453T	S91I	—
IIHGaC	32	1	1	4	2	S153L	—	—	E466K
IIHGaD	32	8	1	0.25	2	E157K	—	—	D426N
IIHGaA	>128	64	8	8	16	S153L	—	S91I	—
IIHGaC	>128	64	16	16	64	S153L	—	S91I	E466K

[a] CIP, ciprofloxacin; SPX, sparfloxacin; LVX, levofloxacin; MXF, moxifloxacin; GEM, gemifloxacin; GAT, gatifloxacin.
[b] *M. hominis* positions GyrA 152, 153, 157, and 163, GyrB 450 and 453, ParC 84 and 91, and ParE 426, 447, and 466 correspond to *E. coli* coordinates GyrA 82, 83, 87, and 93, GyrB 450 and 453, ParC 73 and 80, and ParE 420, 441, and 460.
[c] *M. hominis* PG21 is the parental strain. Agar-selected mutants are designated by a prefix corresponding to the selection step (I, II, or III) followed by initials of the species (H) and of the selector fluoroquinolone (L, levofloxacin; M, moxifloxacin; G, gemifloxacin; Ga, gatifloxacin). Only clones with significantly increased MICs and QRDR mutations are shown. See footnote *a* for three-letter drug abbreviations.
[d] —, identical to the reference strain.

M. hominis. These mutants exhibited cross-resistance for fluoroquinolones, with the MICs depending on the number of mutations and on the altered positions in the QRDRs. Moxifloxacin, gemifloxacin, and gatifloxacin but not ciprofloxacin and levofloxacin stayed active against *M. pneumoniae* or *M. hominis* mutants with one mutation. No efflux mechanism was detected for *M. pneumoniae* and *M. hominis* mutants in the presence of reserpine.

For *M. pneumoniae*, mutations were found at positions described as hot spots of fluoroquinolone resistance, such as GyrA 99 (83 for *Escherichia coli*), GyrB 443 and 464 (426 and 447 for *E. coli*), and ParC 83 and 87 (80 and 84 for *E. coli*) (7). Mutations ParC 81 (78 for *E. coli*) and GyrB 483 (466 for *E. coli*) were described previously for *E. coli* and *Staphylococcus aureus* (7) and for *Proteus mirabilis* and *Pseudomonas aeruginosa* (7, 13), respectively. Only position ParE 449 (439 for *E. coli*) was not previously associated with quinolone resistance in other bacteria. In *M. hominis*, besides the mutations previously found either in this mycoplasma or in other bacteria (7), several new mutations were found in this study, at positions GyrA 163 (93 for *E. coli*), GyrB 450 and 453 (same *E. coli* numbering), and ParE 447 (441 for *E. coli*). However, what real effect these new mutations have on quinolone susceptibility of both mycoplasmas has yet to be determined. In *M. pneumoniae*, *gyrB* mutations seem to be predominant over *gyrA* mutations, in contrast to the case for *M. hominis* and *Ureaplasma* spp. (1).

As in *M. hominis*, distinct fluoroquinolones seem to have different primary targets in *M. pneumoniae*. Thus, according to our genetic studies with *M. pneumoniae*, sparfloxacin and ciprofloxacin selected mutants with a single modification in *parC* alone, while levofloxacin and moxifloxacin selected single mutants with a mutation in *gyrA* or *gyrB* alone. Gemifloxacin selected single mutants with a modification in either *gyrA* or *parC*, in support of dual activity on both DNA gyrase and topoisomerase IV in *M. pneumoniae*, as in *Streptococcus pneumoniae* (9). It should be noted that the same fluoroquinolone did not have the same preferential target in both mycoplasmas species. For instance, sparfloxacin targeted primarily topoisomerase IV in *M. pneumoniae* (Table 1) but DNA gyrase in *M. hominis* (2, 8). For *M. hominis*, first-step mutants that were selected with newer fluoroquinolones, levofloxacin and gatifloxacin, had mutations in *parC* or *parE*. Here again, first-step mutants of *M. hominis* selected with gemifloxacin harbored mutations in either *gyrA* or *parC*. The preferential targets of moxifloxacin with *M. hominis* and gatifloxacin with *M. pneumoniae* were not elucidated in our study, with no single mutants selected with these compounds.

To our knowledge, this is the first description of fluoroquin-

olone resistance acquired in *M. pneumoniae*. Even though lower mutation rates were obtained with broader-spectrum quinolones, such as moxifloxacin, gemifloxacin, and gatifloxacin, than with ciprofloxacin for both mycoplasma species, all fluoroquinolones tested selected for resistance, confirming the necessity of a judicious use of these compounds in order to limit development of resistance.

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