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Comparative Study of the Mutant Prevention Concentrations of Moxifloxacin, Levofloxacin, and Gemifloxacin against Pneumococci▽†

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We tested the propensity of three quinolones to select for resistant *Streptococcus pneumoniae* mutants by determining the mutant prevention concentration (MPC) against 100 clinical strains, some of which harbored mutations in type II topoisomerases. Compared with levofloxacin and gemifloxacin, moxifloxacin had the lowest number of strains with MPCs above the susceptibility breakpoint ($P < 0.001$), thus representing a lower selective pressure for proliferation of resistant mutants. Only moxifloxacin gave a 50% MPC (MPC_{50}) value (1 μg/ml) within the susceptible range.

The only three quinolones currently approved to treat community-acquired respiratory tract infections in adults are levofloxacin (500 and 750 mg), moxifloxacin (400 mg), and gemifloxacin (320 mg). When free area under the curve (AUC)/MIC data are compared, moxifloxacin and gemifloxacin yield similar values and are superior to levofloxacin (15, 16). These pharmacokinetic/pharmacodynamic considerations relate to efficacy against the susceptible portion of the bacterial population (9, 10). Another criterion, the mutant prevention concentration (MPC), may also impact clinical efficacy by relating to the resistant mutant subpopulations. The MPC, which is the MIC of the least susceptible resistant mutant subpopulation, represents a threshold above which the selective proliferation of the resistant mutant subpopulation is expected to rarely occur. Using this method, Blondeau and colleagues (4) proposed that moxifloxacin and gatifloxacin (a drug no longer available) are more active than levofloxacin in the treatment of pneumococcal pneumonia. Since the least susceptible mutant is usually a drug target mutant, MPC values can be regarded as a measure of the interaction of the quinolone with the mutant target enzyme. However, clinical isolates may contain additional efflux mechanism and/or permeability alterations that potentially may raise the MPC. Thus, measurement of MPC with clinical isolates and integrating that information with pharmacokinetics is important for comparing compounds.

In the current study, the MIC and MPC values for 100 recent clinical pneumococcal isolates, each with different β-lactam, quinolone, and macrolide phenotypes, were determined for levofloxacin, moxifloxacin, and gemifloxacin. All quinolone-nonsusceptible (intermediate as well as resistant) strains, as well as 13 randomly selected quinolone-susceptible strains were tested for the presence of mutations in the quinolone resistance-determining regions (QRDRs) of the *gyrA*, *gyrB*, *parC*, and/or *parE* genes.

MATERIALS AND METHODS

MIC and MPC determination. The initial determinations of the MICs for all strains were made using standard agar dilution methodology (5). MICs were also tested during MPC determinations that were performed as follows. Starter cultures were generated by inoculating 12 blood Trypticase soy agar (TSA) plates (BD Diagnostics, Sparks, MD) with pure cultures of each test isolate and incubated overnight at 35°C in air. The cells were then transferred from plates and suspended in 12 ml of 0.9% sterile saline. These cultures were concentrated by centrifugation (3,000 × *g* for 20 min at 25°C) and resuspended in 800 μl of 0.9% sterile saline. The total number of CFU/ml was determined on drug-free blood agar plates (BD Diagnostics). Each quinolone was incorporated in 2-fold multiples of the initial MIC (ranging 0.25× to 16× MIC) into Mueller-Hinton plates supplemented with 5% sheep blood (BD Diagnostics); the plates were stored at 4°C for a maximum of 7 days.

For MPC experiments, 50-μl aliquots containing $\geq 10^{10}$ CFU were applied to duplicate plates containing antibiotic. *Streptococcus pneumoniae* ATCC 49619 was included as a control. Inoculated plates were incubated at 35°C in 5% CO_2 for 48 h and examined for growth. Plates with growth at or above the determined MIC were analyzed as follows. If >300 colonies (confluent growth) were observed, colonies from six different areas of the analyzed plate were subcultured on a drug-free plate (BD Diagnostics), and pneumococcal identification was confirmed by optochin susceptibility testing. Optochin-susceptible bacteria were suspended in 0.9 ml cation-adjusted Mueller-Hinton broth (BD Diagnostics) and retested by agar dilution; those organisms that retested at values at or above the antimicrobial concentration used in the initial selection were labeled mutants. If ≤300 colonies were observed and colonies exhibited different morphologies, one of each colony type was subcultured on a drug-free plate (BD Diagnostics) and tested for optochin susceptibility. After confirmation of a pure culture, organisms were examined by repeat agar dilution testing at drug concentrations equivalent to those used for the initial selection. Agar dilution determination, confirming the presence or absence of mutants, was included to eliminate bacterial overcrowding, which may cause an increase in the drug concentration required to prevent isolation of mutants (4, 12). If hazy growth or a thin film was observed, making accurate reading of endpoints difficult, the plates were washed with 1-ml portions of 0.9% sterile saline, and suspensions were streaked onto a fresh blood agar plate to confirm minimal/no growth, thus obtaining the true MPC (4). If growth on drug-free plates occurred, colonies were tested by optochin susceptibility followed by agar dilution determination to confirm the presence or absence of mutants at or above the concentration used to select colonies.

After the cultures were retested for mutants, the MPC was determined and defined as the lowest fluoroquinolone concentration that prevented growth of resistant mutants for each drug-isolate combination. The ratio of MPC to MIC was then calculated.

QRDR determination. PCR was used to amplify fragments of *gyrA*, *gyrB*, *parC*, and *parE* genes using primers and cycling conditions described previously (17,

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TABLE 1. Percentage of MIC and MPC values at CLSI breakpoints for all strains tested[a]

Drug and parameter	% of strains in each breakpoint category		
	Susceptible	Intermediate	Resistant
Levofloxacin			
MIC	66	3	31
MPC	18	42	40
Moxifloxacin			
MIC	71	6	23
MPC	57	9	34
Gemifloxacin			
MIC	67	4	29
MPC	14	28	58

[a] The CLSI breakpoints (6) are as follows for the three drugs: for levofloxaxin, ≤2 μg/ml for susceptible, 4 μg/ml for intermediate, and ≥8 μg/ml for resistant; for moxifloxacin, ≤1 μg/ml for susceptible, 2 μg/ml for intermediate, and ≥4 μg/ml for resistant; for gemifloxacin, ≤0.125 μg/ml for susceptible, 0.25 μg/ml for intermediate, and ≥0.5 μg/ml for resistant.

21). The amplification products were sequenced directly using a CEQ8000 genetic analysis system (Beckman Coulter Inc., Fullerton, CA).

DI calculation. The dosing interval (DI) was calculated by DI = $t_{1/2} \times \log_2(C_{max}/\text{MPC})$, where $t_{1/2}$ is the half-life of the serum drug concentration, C_{max} is the maximum serum concentration, and MPC is the mutant prevention concentration.

Statistical analysis. The chi-square goodness-of-fit test was performed to evaluate the different values for groups susceptible to moxifloxacin, levofloxacin, and gemifloxacin. A P value of <0.05 was considered significant. Determination coefficients (R^2) were calculated to evaluate correlation between MPC and MPC provisional values as defined by Blondeau et al. (4).

RESULTS AND DISCUSSION

The prevalence of fluoroquinolone resistance in *de novo* clinical pneumococcal isolates is currently <5% (8). Higher fluoroquinolone resistance rates in our study reflect preselection of strains with mutations in the QRDRs. Using Clinical and Laboratory Standards Institute (CLSI) susceptibility criteria (6), 71 of the 100 strains were susceptible to moxifloxacin, whereas 66 and 67 strains were susceptible to levofloxacin and gemifloxacin, respectively (Table 1). It is worth mentioning that the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints of moxifloxacin are lower than those defined by CLSI (>0.5 μg/ml), and the levofloxacin breakpoints are the same (6; EUCAST website http://www.srga.org/eucastwt/MICTAB [clinical breakpoints accessed 7 April 2008]). Using EUCAST breakpoints, 70 strains in our study are moxifloxacin susceptible, whereas 71 are susceptible by CLSI criteria. The setting of breakpoints for fluoroquinolones and other drugs is a contentious matter, and their clinical significance is sometimes difficult to evaluate (7, 20).

For moxifloxacin, 57 strains had MPCs at or below the moxifloxacin susceptible breakpoint of ≤1 μg/ml. Of the 57 strains, 18 were levofloxacin susceptible, 37 were intermediate, and 2 were resistant, whereas 13 strains were gemifloxacin susceptible, 25 were intermediate, and 19 were resistant. In contrast, the MPCs of only 18 and 14 strains were at or below the susceptible breakpoints of levofloxacin (≤2 μg/ml) and gemifloxacin (≤0.125 μg/ml), respectively ($P < 0.001$) (Table 1).

Provisional MPCs and their respective final MPC values correlated well ($R^2 = 0.72$), and provisional and final MPC_{50}/MPC_{90} values (MPC_{50} is the 50% MPC and MPC_{90} is the 90% MPC) were identical for each drug. These facts indirectly indicate that provisional and final MPC values are interchangeable. We focused our analysis only on final MPC values. All raw data are presented in the supplemental material.

TABLE 2. MIC_{50}, MIC_{90}, MPC_{50}, and MPC_{90} values for all 100 isolates tested

Drug	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MPC_{50} (μg/ml)	MPC_{90} (μg/ml)	MPC_{50}/MIC_{50} ratio	MPC_{90}/MIC_{90} ratio
Levofloxacin	2	32	4	64	2	2
Moxifloxacin	0.5	4	1	16	2	4
Gemifloxacin	0.125	1	0.5	4	4	4

Blondeau and coworkers (4) described the crowding effect of the inoculum, which may be observed if 10^{10} CFU were applied on the plate with the antibiotic concentration used for selection. To investigate such phenomena, the MPC/MIC ratio was tested when 10^8 cells were applied to a plate with an antibiotic concentration 1 dilution lower than the provisional MPC (MPC_{pr}). We determined the presence/absence of resistant mutants by agar dilution determination in all cases when growth occurred at or above the antibiotic concentration used for selection. We believe that such determination would better reflect the real value of MPC at the 10^{10} organism concentration.

The MPC/MIC ratio defines the concentration range in which resistant mutant subpopulations are selectively amplified, with the lower values suggesting a better ability to prevent mutant emergence (4, 13, 23, 25). In the current study, moxifloxacin and levofloxacin MPC/MIC ratios were within the same range for most strains; >80% of strains had ratios of 2 to 4. Gemifloxacin showed a slightly extended range, with most strains having a ratio of 2 to 8.

When the MIC of the isolate as an entire bacterial population exceeds the susceptibility breakpoint, it is considered clinically resistant. Susceptible isolates may have subpopulations for which the MIC, i.e., MPC, exceeds that breakpoint. Resistance is expected to emerge more readily from such isolates than from isolates with the MPC below the breakpoint. MIC_{50}, MPC_{50}, MIC_{90}, and MPC_{90} values (μg/ml) are listed in Table 2. The MPC_{50} values for levofloxacin (4 μg/ml) and gemifloxacin (0.5 μg/ml) were higher than the corresponding susceptible breakpoints of ≤2 μg/ml and ≤0.125 μg/ml, respectively. In contrast, the MPC_{50} of moxifloxacin was below the susceptibility breakpoint of ≤1 μg/ml. The MPC_{90} values for all three drugs were in the MIC range categorized as resistant (Table 2). In agreement with our findings, Blondeau and colleagues (4) reported moxifloxacin and levofloxacin MPC_{50} values of 1 μg/ml and 4 μg/ml, respectively, as well as Hansen and colleagues (11) who have reported similar (1-fold-lower) MIC_{50}s of moxifloxacin, gemifloxacin, and levofloxacin (Table 2).

Out of 100 tested strains, 79 (all 66 nonsusceptible and 13 randomly selected susceptible isolates) were screened for QRDR mutations in *gyrA*, *gyrB*, *parC*, and *parE* genes (see Table S4 in the supplemental material). Ten strains had no changes in any of the genes tested and were susceptible to all fluoroquinolones studied; the remaining 69 strains had

TABLE 3. QRDR alternations versus MIC and MPC values in parental strains and first-step mutants

Strain	Drug used for mutant selection	Parental MIC (μg/ml)	MPC (μg/ml)	QRDR change(s)			
				Parental strain			GyrA in the selected mutant
				GyrA	ParC	ParE	
1072	Levofloxacin	32.0	256.0	$S_{81}F$	$S_{79}Y$	$I_{460}V$	$S_{82}P$
	Gemifloxacin	1.0	8.0	$S_{81}F$	$S_{79}Y$	$I_{460}V$	$S_{82}P$
	Moxifloxacin	8.0	64.0	$S_{81}F$	$S_{79}Y$	$I_{460}V$	$E_{85}A$
1149	Levofloxacin	8.0	>64.0		$S_{79}Y$		$S_{81}F$
	Gemifloxacin	0.5	>8.0		$S_{79}Y$		$S_{81}F$
	Moxifloxacin	0.5	>8.0		$S_{79}Y$		$S_{81}F$
1151	Levofloxacin	4.0	32.0		$D_{83}N$ $K_{137}N$	$I_{460}V$	$S_{81}Y$
	Gemifloxacin	1.0	8.0		$D_{83}N$ $K_{137}N$	$I_{460}V$	$S_{81}F$
	Moxifloxacin	1.0	>8.0		$D_{83}N$ $K_{137}N$	$I_{460}V$	$S_{81}F$
3321	Levofloxacin	2.0	32.0		$S_{79}F$ $K_{137}N$		$S_{81}Y$
	Gemifloxacin	0.125	2.0		$S_{79}F$ $K_{137}N$		$S_{81}Y$
	Moxifloxacin	0.5	>8.0		$S_{79}F$ $K_{137}N$		$S_{81}Y$
1058	Levofloxacin	8.0	64.0		$S_{79}F$ $K_{137}N$	$I_{460}V$	$S_{81}F$
	Gemifloxacin	0.125	1.0		$S_{79}F$ $K_{137}N$	$I_{460}V$	$E_{85}G$
	Moxifloxacin	0.5	>4.0		$S_{79}F$ $K_{137}N$	$I_{460}V$	$S_{81}Y$
1065	Levofloxacin	4.0	32.0		$S_{79}Y$	$I_{460}V$	$S_{81}Y$
	Gemifloxacin	0.125	4.0		$S_{79}Y$	$I_{460}V$	$E_{85}K$
	Moxifloxacin	0.5	>8.0		$S_{79}Y$	$I_{460}V$	$E_{85}K$
1135	Levofloxacin	16.0	128.0	$S_{81}A$	$S_{79}Y$		$S_{81}V$
	Gemifloxacin	0.5	4.0	$S_{81}A$	$S_{79}Y$		$S_{81}A$
	Moxifloxacin	2.0	32.0	$S_{81}A$	$S_{79}Y$		$S_{81}V$

changes in one or more genes. Thirty had substitutions in GyrA ($A_{147}G$ or $S_{81}A/C/F/Y$ or $E_{85}K$), whereas 23 had single ParC mutations ($S_{79}F/Y$ or $D_{83}N$ or $K_{137}N$), and 18 had double or triple substitutions in ParC ($S_{79}F/Y$ $D_{83}N$ or $S_{79}F/Y$ $K_{137}N$). One strain had a substitution in GyrB ($N_{473}D$), and 59 strains had substitutions in ParE ($D_{435}N$ or $I_{460}V/N$ or $V_{461}I$). Mutations in GyrA and ParC have been reported to have the most impact on MIC and MPC (1, 4, 23). In this study, 43 strains had changes in ParC and/or GyrA. Moxifloxacin had the lowest MPCs for strains with a single GyrA mutation plus double ParC mutations (14/43) and for one strain with single mutations in GyrB and ParC (1/43). The MPCs for moxifloxacin and gemifloxacin were similar for strains with a single ParC mutation (9/43) and for a strain with a triple ParC change. Gemifloxacin exhibited the lowest MPCs for strains with single mutations in GyrA (2/43), double mutations in ParC (3/43), and strains with mutations in GyrA plus a single ParC mutation (14/43). Levofloxacin had the highest MPCs for all the previously mentioned strains.

When the strains were stratified according to the individual breakpoints, 71 strains were susceptible to moxifloxacin (MIC $\leq$ 1 μg/ml). The 29 moxifloxacin-non-susceptible strains all had MPCs of $\geq$8 μg/ml and harbored GyrA mutations. In contrast, the 69 levofloxacin-susceptible and -intermediate strains (MICs $\leq$ 4 μg/ml) harbored ParC and/or ParE mutations. Of the 29 strains harboring GyrA mutations, 27 were resistant to gemifloxacin, and the MPCs for these strains were $\geq$2 μg/ml. There were two levofloxacin- and two gemifloxacin-resistant strains (total of 4 strains), which had no mutation in GyrA, but whose MPC showed a greater than 4-fold increase of concentration used for selection (mutants). These data strongly suggest that GyrA is the major target for all fluoroquinolones tested by our methodology and that GyrA amino acid alternations resulted in the high levels of resistance.

An additional 21 mutants, derived from a panel of seven parental strains with a diverse pattern of preexisting QRDR mutations, were selected and subsequently analyzed for acquisition of additional mutations in the QRDRs (Table 3). The only mutations detected were in *gyrA*; these mutations were in addition to the preexisting alterations in *gyrA*, *parC*, and *parE*. The GyrA alterations ($S_{81}F/Y$, $S_{82}P$, and $E_{85}A$) resulted in MPC increases of $\geq$8$\times$ MICs for all three fluoroquinolones (Table 3). These results further suggest that GyrA was the most crucial target for development of high-level resistance reflected in high MIC and MPC values for these three fluoroquinolones and the only target sensitive to prolonged antibiotic pressure in MPC experiments. These results are in agreement with previous findings (1, 4, 18, 23, 25, 27). No clear relationship was found between the MPCs or their ratios to the MICs and the existence of *gyrB*, *parC*, or *parE* mutations.

Antibacterial activity is assessed by considering MICs together with pharmacodynamic and pharmacokinetic parameters (e.g., time above MIC and the AUC/MIC ratio). For quinolones, free AUC/MIC is the necessary criterion, with a minimum number of 25 necessary for clinical efficacy. Controversy exists surrounding the magnitude of the AUC/MIC ratio required to maximize the clinical outcome, and many authors have shown that *S. pneumoniae* clearance from *in vitro* models

by fluoroquinolones occurs at an AUC/MIC ratio of 30 to 50. In immunocompromised patients on intravenous therapy, a ratio of at least 100 is required (7, 19) The exact role of protein binding in calculation of the above number is also a matter of discussion (7, 19). It is important to note that when the free AUC/MIC of moxifloxacin is examined, a pneumococcal susceptibility breakpoint of ≤2 μg/ml seems entirely feasible. If a moxifloxacin breakpoint of ≤2 μg/ml would have been used in the current study, moxifloxacin MPCs would have been within the susceptibility range for 66 of the 100 strains. The potential value of pharmacokinetic/pharmacodynamic criteria seem not to dictate a higher susceptibility breakpoint for levofloxacin of ≤2 μg/ml (which has, even with the 750-mg daily dose, a lower free AUC/MIC) than moxifloxacin (2). There are conflicting opinions regarding the importance of protein binding in calculation of pharmacodynamic parameters (9). As a result, the true antibacterial effect of highly protein-bound agents may be seriously understated. We are aware of the possible errors provided by such simulation (22).

For MPC to be a therapeutically useful parameter, the value of serum drug concentrations should be above the MPC even after administration of drug doses that are safe for patients (14, 24, 26). However, the latter criteria depend on whether one is looking at the problem from a public health or individual patient point of view. With the former, the issue is whether resistance will eliminate the antibiotics for future generations; with the latter, the issue is patient safety if the probability of resistance in that patient is low at the time of therapy. Moreover, it has been shown that antibiotic concentrations should be above the MPC for a certain portion of the dosing interval (10); specifically, Blondeau et al. (3) reported that moxifloxacin and gemifloxacin achieved drug concentrations in excess of the MPC_{90} for the necessary time interval T ($T \geq 12$ h) to ensure through and rapid killing, in contrast to levofloxacin ($T \leq 4$ h).

To assess the potency of each tested fluoroquinolone, we calculated a DI (23) based on recommended dosage (multiple doses) and pharmacokinetic parameters (C_{max} and $t_{1/2}$), each based on parameters in the products' prescribing information. The calculated DI reflects the number of strains that the dosage regimen will maintain serum drug concentration above the MPC for ≥12 h. Moxifloxacin (400 mg) showed a DI of >12 h for 77 strains. For levofloxacin (750 mg), 66 strains had a DI of >12 h. Gemifloxacin (320 mg) had a DI of >12 h for 71 strains. Hansen et al. (11) have also reported moxifloxacin to be potentially most effective compared to gemifloxacin, gatifloxacin, and levofloxacin at restricting development of resistance despite gemifloxacin having the lowest MIC and MPC.

Moxifloxacin has a maximum serum concentration of 4.5 μg/ml, about twice that of the MPC for 66% of strains tested, including quinolone-resistant strains. Since the half-life of moxifloxacin is 12 h and the time to maximum concentration of drug in serum (T_{max}) is 0.75 to 3.5 h (24), daily dosing should keep concentrations of moxifloxacin in serum above the MPC for most of the treatment time for susceptible strains and strains with one-step mutations. Levofloxacin and gemifloxacin may require higher doses than that currently approved to attain the same potency.

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