Mutant Selection Window in Levofloxacin and Moxifloxacin Treatments of Experimental Pneumococcal Pneumonia in a Rabbit Model of Human Therapy

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For some pneumococci the fluoroquinolone MICs are low but the mutant prevention concentrations (MPCs) are high; this difference defines in vitro the mutant selection window (MSW). We investigated in vivo the bacterial reduction and the occurrence of resistant mutants with moxifloxacin (MFX; 400 mg once daily) or levofloxacin (LVX; 500 mg twice daily) in treatments similar to those in humans with experimental pneumonia due to pneumococci (expPP) exhibiting various MICs and MPCs. The MIC/MPC for MFX and LVX and genotypes were as follows: strain 16089, 0.125/0.125 and 0.5/0.5 (wild type); strain MS1A, 0.25/0.25 and 1/2 (efflux); strain MS2A, 0.25/4 and 1.75/28 (*parC79*); strain MR3B4, 0.25/4 and 2/32 (*parC79*); strain M16, 0.5/2 and 8/32 (*parC83*); strain Gyr-1207, 1.5/3 and 8/16 (*gyrA*); and strain MQ3A, 4/4 and 16/64 (*parC* and *gyrA*). Both drugs were efficient with wild type-expPP, but only MFX was efficient with efflux-expPP. No bacterial reduction was observed for *parC*-expPPs due to mutants observed in 18 to 100% of animals, depending on the strain and the drug tested. These mutants showed unbound area under the concentration-time curve and MICs of from 50 to 164 for MFX. The in vivo pharmacodynamic boundaries of the MSW were different for MFX and LVX. We conclude that, after LVX or MFX treatment, mutants occur in vivo if there is a preexisting *parC* mutation, since the drug concentrations fall below the MPCs of these strains. Since the MPC determination cannot be routinely determined, these phenotypes or genotypes should be detected by simple tests to guide the therapeutic options.

Pneumonia remains a leading cause of death throughout the world, and *Streptococcus pneumoniae* is the most common cause of significant morbidity and mortality (36, 53–55, 70). The prevalence of antibiotic-resistant *S. pneumoniae* has increased over the last decade (18, 25, 28, 44, 59, 72), and beta-lactam resistance could be associated with increased morbidity and mortality (20, 36, 48, 51, 57). Newer fluoroquinolones, because of their antipneumococcal activities, are also part of the therapeutic arsenal for pneumococcal pneumonia. Among these compounds, levofloxacin (LVX) and moxifloxacin (MFX) were shown to be as effective as comparators in phase III studies (10, 21, 22, 56, 61). However, because of the very low frequency of pneumonia due to strains with low-level fluoroquinolone resistance (efflux and/or mutation in the topoisomerase IV gene), these studies cannot provide measurements of the real efficacy of these fluoroquinolones versus infections due to such strains.

Recently, two major observations have emerged: first, failures of LVX treatment for pneumococcal pneumonia have been described in patients, most of whom were previously exposed to fluoroquinolones (16, 24, 37, 39, 64, 69, 71; N. Fishman, B. Suh, L. Weigel, B. Lorber, S. Gelone, A. Truant, T. Gootz, J. Christie, and P. Edelstein, Abstr. 39th, Intersci. Conf. Antimicrob. Agents Chemother., abstr. 825, p. 111,

1999) and, second, an increasing rate of low-level fluoroquinolone-resistance that occurs in some areas of the world (9, 11, 25, 28, 30, 34, 44, 60, 73).

New chemical developments on fluoroquinolones, especially C-8-OMe-fluoroquinolones, have led to a stronger intrinsic in vitro activity associated with a low frequency of mutation (1, 15, 19). However, further information concerning the in vivo efficacy of new fluoroquinolones is needed. Especially, the risk of in vivo mutations has to be investigated, taking into account the high in vitro rate of mutation of pneumococci when exposed to quinolones (1, 6, 41, 42, 45, 46, 55, 66, 68, 74) compared to beta-lactam agents.

The aim of the present study was to investigate both the efficacy of LVX and MFX and the pharmacodynamic (PK-PD) conditions associated with the eventual occurrence of resistant mutants in a simulated human-like treated experimental pneumonia induced with pneumococcal strains with various degrees of susceptibility to fluoroquinolones. For this investigation, in addition to the conventional PK-PD parameters using MIC, we also applied in vivo the novel in vitro concept of the mutant selection window (MSW), which is defined as the zone between MIC and mutant prevention concentration (MPC) (5, 41, 76, 78) and which provides a means for defining the ability of antibiotics in preventing the emergence of mutants.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and antibiotics. The bacteria used in the experiments are listed in Table 1. These strains include strain 16089, a clinical strain of *Streptococcus pneumoniae*, isolated from a patient with pneumonia and

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TABLE 1. In vitro susceptibilities of *S. pneumoniae* strains to antibiotics and mutations identified in the QRDRs (*parC*, *parE*, *gyrA*, and *gyrB*)

Strain	Concn (mg liter^{-1})					SI		Efflux[a]	QRDR genotypic mutations[b]			
	MIC			MPC								
	CIP	LVX	MFX	LVX	MFX	LVX	MFX		*parC*	*parE*	*gyrA*	*gyrB*
16089	0.5	0.5	0.125	0.5	0.125	1	1	−	−	−	−	−
MS1A	2	1	0.25	2	0.25	2	1	+	−	−	−	−
MS2A	8	1.75	0.25	28	4	16	16	+	Ser-79→Phe	−	−	−
MR3B4	10	2	0.25	32	4	16	16	+	Ser-79→Tyr	−	−	−
M16	64	8	0.5	32	2	4	4	+	Asp-83→Asn	−	−	−
Gyr-1207	6	8	1.5	16	3	2	2	−	−	−	Ser-81→Phe	−
MQ3A	>64	16	4	64	4	4	1	+	Ser-79→Tyr	−	Ser-81→Phe	−

[a] Efflux reserpine-CIP.
[b] −, No mutation.

kindly provided by the Centre National de Référence des Pneumocoques (Dr Geslin); five ciprofloxacin (CIP)-resistant laboratory derivatives of strain 16089 (MS1A, MS2A, MR3B4, M16, and MQ3A), selected by serial exposure of the 16089 parent to this drug (14); and a clinical strain, Gyr-1207, kindly provided by G. Doern (18). All of these strains were resistant to penicillin (MIC = 4 mg liter^{-1}). Bacteria were grown in 5% CO_2 either in brain heart infusion broth (BioMérieux, Marcy l'Etoile, France) or on sheep blood agar plates (Bio-Mérieux). Bacterial stocks were kept at −70°C in a 15% (vol/vol) glycerol-supplemented brain heart infusion broth.

LVX and MFX were provided by Aventis (Paris La Défense, France) and Bayer AG (Puteaux, France), respectively. The drugs were reconstituted according to the manufacturer's instructions. Stock solutions were used as fresh preparations.

In vitro susceptibility studies. (i) MIC determination. The MICs of LVX and MFX for the various isolates were determined by the standard dilution method in agar (12).

(ii) MPC determination. For MPC determination (5), 200 μl of a culture containing ≥10 log$_{10}$ CFU/ml was applied to tryptic soy agar plates containing 5% sheep blood and drugs at various concentrations. MPCs were read after 24 and 48 h of incubation at 37°C in 5% CO_2. MPCs were recorded as the lowest antibiotic concentration that prevented bacterial colony formation at 48 h. All determinations were done in triplicate, and the results were identical. The absence of visible growing bacteria at 24 h allowed us to eliminate the presence of subpopulations in the inoculum.

The selection index (SI) was calculated for each strain as the MPC/MIC ratio.

Evaluation of efflux for CIP. MIC determination was done in parallel both with or without an efflux inhibitor (10 μg of reserpine/ml) (8, 27, 47). Efflux was considered as present when a ≥2-fold reduction of the MIC was observed.

PCR amplification of quinolone resistance-determining regions (QRDRs) and DNA sequencing. Genomic DNA was isolated from bacterial strains and used as the template in PCR amplification of the QRDRs of *parC*, *parE*, *gyrA*, and *gyrB* genes as previously described (58). Nucleotide sequencing (Génôme Express S.A., Grenoble, France) was carried out with an automated sequencer (ABI Prism 377 DNA sequencer) with published primers (52).

Preparation of the inoculum. Before each animal experiment, several *S. pneumoniae* strains from one aliquot (per strain) were inoculated into brain heart infusion broth, cultured on agar plates, and incubated for 24 h at 37°C in 5% CO2. Twenty-five to thirty colonies were taken and inoculated into 9 ml of brain heart infusion broth, incubated for 6 h at 37°C, and then cultured on agar plates for 18 h at 37°C in 5% CO_2. This culture was diluted in physiologic saline in order to obtain final concentrations of 10 log$_{10}$ CFU/ml. No adjuvant was used. These concentrations were first determined by using optical density measurements, in reference to a standard curve, and then confirmed by using successive dilution cultures.

Animals. Male New Zealand White rabbits (body weight, 2.5 to 3 kg) were obtained from Elevage Scientifique des Dombes (Romans, France). Animals were not immunosuppressed and were virus antibody and specific pathogen free. They were placed in individual cages and were nourished ad libitum with drinking water and feed according to current recommendations.

Production and humanized treatment of experimental pneumococcal pneumonia in rabbits. Production of pneumonia in immunocompetent rabbits and the installation of the central venous catheters were performed as previously described (14, 62). Briefly, 24 h after jugular catheterization, bacterial pneumonia was induced by endobronchial challenge of the animals with 0.5 ml of saline containing 10 log$_{10}$ CFU/ml of either tested strain (day-to-day variability of 0.3 log$_{10}$ CFU. Treatment was started 5 h after bacterial challenge and lasted for 2 days. Antibiotics were delivered through the first central venous catheter with changing infusion rates obtained by a computer-controlled electric pump and at doses that simulated antibiotic kinetics observed in human serum, as follows: (i) 500 mg of LVX given intravenously (i.v.) twice a day (C_{max} = 6 mg/liter; area under the concentration-time curve from 0 to 24 h [AUC$_{0-24}$] = 50 mg · h · liter^{-1}), (ii) 400 mg of MFX given i.v. once a day (C_{max} = 4.5 mg/liter; AUC$_{0-24}$: 40 mg h liter^{-1}).

Pharmacokinetic analysis. For each animal, the concentrations of antibiotics in the serum were determined on iterative blood samples, obtained through the second central catheter. LVX and MFX concentrations were determined by a disk plate bioassay method with antibiotic medium II (Difco Laboratories) and *Escherichia coli* NIJJHC2 as the indicator organism. The limit of detection was 0.4 mg/liter. Standard curves were established with solutions (progression from 0.5 to 7 mg/liter) in serum. The linearity of the standard curves used for disk plate bioassays was at least 0.98 (r^2). The serum samples were diluted in serum water to ensure that their concentrations would be within the range of those on the standard curve. The standard samples were assayed for each experiment, and concentrations were assayed in duplicate. The between- and within-day coefficients of variation for replicates were equal to 5 and 8%, respectively. The level of plasma protein binding was determined ex vivo for LVX and MFX by a membrane filtration method (67). Pharmacokinetics data were analyzed by using Kinetica software (Innaphase, Philadelphia, Pa.).

Evaluation of infection. The rabbits were anesthetized and sacrificed 2 h after the end of the antibiotic infusion.

(i) Bacterial content in the lungs and spleen. The spleen and each pulmonary lobe were weighed and homogenized in sterile serum saline. Bacteria were counted in a sample of this crude homogenate by plating 10-fold dilutions on sheep blood agar and incubating the plates for 24 h at 37°C. Bacterial concentrations in each lobe and in the spleen were determined after adjusting for weight. The threshold value was 1 log$_{10}$ CFU/ml since a large volume of pulmonary homogenate was plated. For statistical comparisons of the difference between the pulmonary bacterial densities, culture-negative lobes were considered to contain 1 log$_{10}$ CFU/g, whereas no carryover effect was found. For each rabbit, the mean pulmonary pneumococcal concentration was calculated according to each lobar bacterial concentration with lobar weight [e.g., mean concentration = Σ (lobar concentration × lobar weight)/Σ (lobar weights)].

(ii) In vivo mutants. In the treated animals and for each lobe or spleen with residual surviving bacteria, emerging mutants were detected by plating 1 ml of the crude tissue homogenate and 10-fold dilutions on sheep blood agar containing two and four times the MIC of the tested strain; a 48-h incubation period was used.

PK-PD analysis. From the individual pharmacokinetics of each treated animal, the following PK-PD parameters (unbound drug) were calculated: C_{max}/MIC, C_{max}/MPC, C_{max}/SI, AUC/MIC, AUC/MPC, AUC/SI, T>MIC, and T>MPC. The examination of the area between the MIC and MPC on one hand and under the concentration-time curve in serum on the other hand made it possible to calculate the following PK-PD parameters of the MSW: time of concentration within the window (T_{MSW}) and AUC$_{MSW}$ (76).

Statistical analysis. The results were expressed as the mean ± the standard deviation. Quantitative variables were compared to Mann-Whitney or analysis of variance and eventually completed by a post hoc analysis by using the Bonferroni test. Percentages were compared by using the chi-square test with the Yates
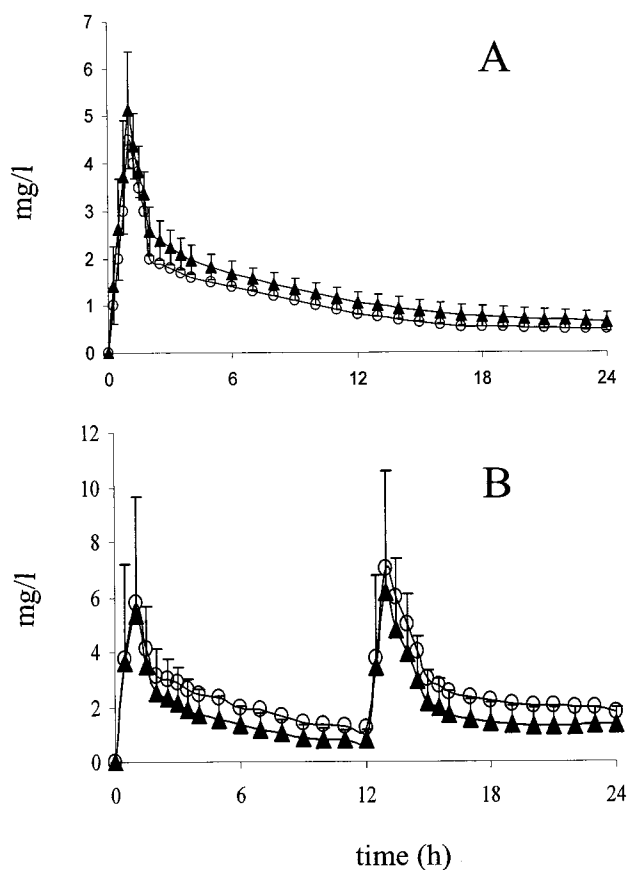
FIG. 1. Pharmacokinetics of MFX (equivalent to the pharmacokinetics in humans at 400 mg once daily [o.d.]) (A) and LVX (equivalent to the pharmacokinetics in humans at 500 mg twice daily [b.i.d.]) (B) in a rabbit model of human therapy. Symbols: ○, desired human concentrations; ▲, obtained concentrations in rabbits.

correction or by using the Fisher exact test. The quantitative relationships between antimicrobial efficacy and each of the PK-PD parameters were determined by using an E_{max} model (Hill formula) or a peak model (Gauss formula), as appropriate, by using SigmaPlot software (version 7.101). To define the PK-PD zones associated with the occurrence of mutants, a qualitative analysis (mutants versus nonmutants) was performed by using classification and regression tree (CART) methodology (7, 43). For all of the tests, a P value of <0.05 was considered significant.

RESULTS

Antimicrobial susceptibility and resistance mutations in the QRDR. The MICs of CIP, LVX, and MFX and the MPCs of LVX and MFX for the seven strains used to induce pneumonia in animals are shown in Table 1. All of these strains, except for strain 16089, were resistant to CIP. LVX and MFX retained their lower MICs against CIP-resistant strains. As expected, the MICs of MFX were lower than those of LVX. Indeed, the first four strains (16089, MS1A, MS2A, and MR3B4) were susceptible to LVX (≤ 2 mg liter^{-1}), and the last three strains (M16, Gyr-1207, and MQ3A) were resistant to LVX, whereas all strains, except for strains Gyr-1207 and MQ3A, were susceptible to MFX (≤ 1 mg liter^{-1}). Although a single *parC* mutation (Ser-79→Phe or Asp-83→Asn) increased the MIC of LVX 2.5 to 16 times, it increased the MIC of MFX only 2 to 4 times. The presence of a *gyrA* mutation (Ser-81→Phe) was associated with a 16-fold increase in the MIC of LVX and a 12-fold increase in the MIC of MFX. However, a double *parC* (Ser-79→Tyr) and *gyrA* (Ser-81→Phe) mutation increased the MICs of both LVX and MFX by a factor of 32. The presence of only one CIP efflux mechanism (strain MS1A) slightly increased the MICs of both LVX and MFX, respectively, from 0.5 to 1 mg liter^{-1} and from 0.125 to 0.25 mg liter^{-1}.

MPCs for MFX were lower than those for LVX. The presence of a *parC* mutation was associated with a greater increase in MPC values than was the presence of a *gyrA* mutation. Of note, the parC-83 mutation (strain M16) led to a lesser increase in the MPCs than did the parC-79 mutations (strains MS2A and MR3B4). Furthermore, the MFX MICs and MPCs were identical for the highly resistant doubly mutated strain (MQ3A); this was not the case for LVX. Finally, the SIs were similar for LVX and MFX.

LVX and MFX simulated human-like treatments. The concentration-time curves of both antibiotics observed for the sera of infected rabbits superimposed on the human pharmacokinetic data as shown in Fig. 1.

There was no difference between rabbits groups as defined by strains (Table 2; $P > 0.4$ for both treatments) with regard to exposure to antibiotics measured by AUC$_{0-24}$.

The protein bindings (from 0.5 to 8 mg/liter) were 45 and 30% for LVX and MFX, respectively.

TABLE 2. Pharmacokinetics of a model of human therapy with LVX (equivalent to 500 mg b.i.d. for 48 h) and with MFX (equivalent to 400 mg o.d. for 48 h) in infected rabbits

Strain	LVX			MFX		
	n	AUC$_{0-24}$ (mg/h liter^{-1})	C_{max} (mg liter^{-1})	n	AUC$_{0-24}$ (mg/h liter^{-1})	C_{max} (mg liter^{-1})
16089	12	42.05 ± 14.28	5.23 ± 1.39	13	35.77 ± 2.98	5.53 ± 1.24
MS1A	6	56.68 ± 22.36	9.38 ± 4.08	14	38.97 ± 5.96	5.69 ± 0.67
MS2A	10	35.55 ± 15.58	3.79 ± 1.90	9	41.30 ± 5.51	6.15 ± 1.85
MR3B4	10	47.39 ± 9.42	5.02 ± 1.44	11	40.32 ± 7.73	5.79 ± 0.93
M16	8	53.93 ± 19.11	6.17 ± 2.69	11	35.82 ± 3.74	5.25 ± 0.48
Gyr-1207		ND^a	ND	10	42.50 ± 9.54	5.97 ± 1.05
MQ3A	7	46.32 ± 25.88	7.60 ± 4.47	5	41.66 ± 5.06	6.30 ± 1.77
Total^b	51	46.45 ± 18.46	6.06 ± 3.20		39.03 ± 6.21	5.75 ± 1.14

^{a} ND, not done.
^{b} No statistically significant difference.

TABLE 3. Pharmacodynamic parameters with the MIC[a]

Strain	Mutant[b]	Mean value ± SD					
		AUC/MIC		C_{max}/MIC		T>MIC	
		LVX	MFX	LVX	MFX	LVX	MFX
16089	−/−	46 ± 15	200 ± 17	5.8 ± 1.5	31 ± 7	36 ± 15	>100
MS1A	−/−	31 ± 13	117 ± 37	5.2 ± 2.4	16 ± 2.5	35 ± 15	>100
MS2A	+/+	11 ± 5	115 ± 15	1.2 ± 0.6	17 ± 5	13 ± 12	>100
MR3B4	+/+	13 ± 3	113 ± 22	1.4 ± 0.4	16 ± 2.6	14 ± 7	>100
M16	−/+	3.7 ± 1.3	50 ± 5	0.4 ± 0.2	7.6 ± 1.2	0	36 ± 5.6
Gyr-1207	−/−	ND	19 ± 4	ND	2.7 ± 0.4	ND	44 ± 16
MQ3A	−/−	1.7 ± 1	7.3 ± 0.8	0.27 ± 0.17	1.1 ± 0.3	0.5 ± 0.7	4.6 ± 2.3

[a] The pharmacodynamic parameters of LVX (500 mg b.i.d.)- and MXF (400 mg o.d.)-treated rabbits with pneumonia exhibiting various susceptibilities to fluoroquinolones were determined and are presented in Tables 3 to 6. In these four tables, all P values as determined by analysis of variance (vertical analysis [treatment between strains]) and the Mann-Whitney test (horizontal analysis [between treatments]) are significant ($P < 0.05$). †, $P > 0.31$. ND, not determined. The numbers of animals per strain and per treatment are indicated in Table 2. Results are expressed as the free fraction of antibiotic concentration.

[b] The occurrence of mutant(s) in treated rabbits (LVX/MFX).

Antimicrobial effects of LVX and MFX in a rabbit model of human therapy on experimental pneumococcal pneumonia. The results are shown in Fig. 2.

(i) Bacterial reduction after treatment. As expected, both antibiotics were strongly effective on pulmonary infection due to the fully susceptible strain 16089. The efficacy of MFX seemed better than that of LVX but was not statistically significant ($P = 0.21$).

For the efflux derivative strain MS1A, in contrast to treatment with LVX, which had little effect, MFX treatment was associated with a significant bacterial reduction (>3 $\log_{10}$ CFU/g) compared to controls without treatment ($P < 0.05$).

As anticipated, both antibiotics were ineffective in rabbits infected with resistant or intermediate strains, i.e., strains M16 and MQ3A (Gyr-1207 strain not tested) for LVX and strains Gyr-1207 and MQ3A for MFX.

For the rabbits infected with *parC* mutant strains, the situation was more difficult to describe. For the LVX-resistant and MFX-susceptible strain M16, which harbored a *parC83* mutation, a significant bacterial reduction was observed with MFX.
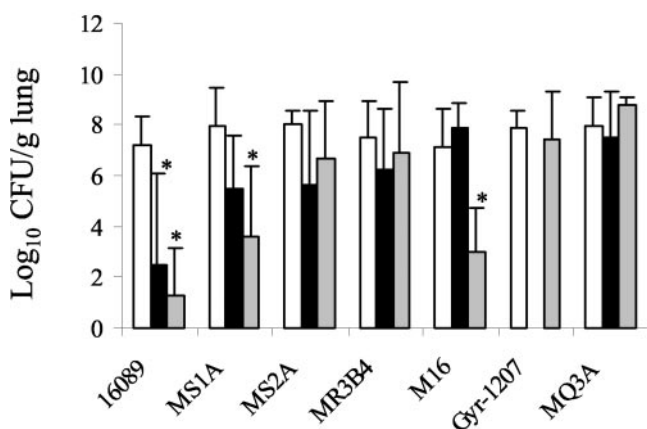

FIG. 2. Pulmonary residual bacterial content ($\log_{10}$ CFU/g) in rabbits infected with pneumococcal strains exhibiting various levels of susceptibility to fluoroquinolones (see Table 1) treated for 48 h in a model of human therapy with LVX (■; equivalent to 500 mg b.i.d.) or MFX (▦; equivalent to 400 mg o.d.) or in a control sample without treatment (□). ✻, $P < 0.05$ versus controls.

For the LVX-borderline-susceptible and MFX-susceptible strains, MS2A and MR3B4, which both harbored a *parC79* mutation, there was no significant bacterial reduction with either antibiotic. Similar results were obtained in the spleen (data not shown). The emergence of mutants was looked for to explain these unexpected results.

(ii) In vivo mutants. Mutants were detected only in rabbits infected with strains already harboring *parC* mutations. No emerging mutants were detected in animals infected with the MQ3A strain. With both *parC79* mutated strains (MS2A and MR3B4), 88 and 100% of the rabbits treated with MFX exhibited resistant mutants, and 20 and 100% of the rabbits treated with LVX exhibited resistant mutants, respectively. The mean pulmonary concentration of these mutants was approximately 5 to 6 $\log_{10}$ CFU/g.

As expected, LVX was completely ineffective in rabbits infected with M16 strain (*parC83*), since this strain was resistant, and no emerging mutant was detected. MFX treatment was associated with the emergence of mutants in 18% of rabbits infected with this latter strain, with a mean pulmonary concentration of approximately 3 $\log_{10}$ CFU/g. Furthermore, fewer rabbits harbored emerging resistant mutants when infected with M16 strain (*parC83*) than when infected by MS2A and MR3B4 strains (*parC79*) (18, 88, and 100%, respectively, $P < 0.001$).

However, there was no significant difference ($P = 0.11$) between LVX and MFX in terms of the percentage of rabbits harboring resistant mutants, when rabbits infected with *parC79* and *parC83* mutated strains were considered as a whole.

All of these mutants acquired a new *gyrA* mutation with a high MIC (LVX MIC = 16 mg liter^{-1} and MFX MIC = 4 mg liter^{-1}). Very similar results were obtained in the spleen (data not shown).

PK-PD analysis. For this analysis, only the free fraction of each antibiotic was considered.

(i) PK-PD with MIC (PK-PD$_{MIC}$). The results of AUC/MIC, C_{max}/MIC, and T>MIC analyses for both antibiotics are shown in Table 3. As expected, the values of all of these parameters decreased when the MICs for both antibiotics increased and were higher for MFX than for LVX (for example, from 7.3 to 200 and from 1.7 to 46 for the AUC/MIC ratios, respectively). When these PK-PD$_{MIC}$ results are compared to

TABLE 4. Pharmacodynamic parameters with the MPC[a]

| Strain | Mean value $\pm$ SD | | | | | |
| | AUC/MPC | | C_{max}/MPC | | T>MPC (h) | |
	LVX	MFX	LVX	MFX	LVX	MFX
16089	46 $\pm$ 15	200 $\pm$ 17	5.8 $\pm$ 1.5	31 $\pm$ 7	36 $\pm$ 15	>100
MS1A	7.7 $\pm$ 3.4	117 $\pm$ 37	1.3 $\pm$ 0.6	16 $\pm$ 2.5	5.2 $\pm$ 4	83 $\pm$ 34
MS2A	0.7 $\pm$ 0.3	7.2 $\pm$ 1	0.07 $\pm$ 0.03	1 $\pm$ 0.3	0	4 $\pm$ 1.3
MR3B4	0.8 $\pm$ 0.16	7 $\pm$ 1.3	0.08 $\pm$ 0.02	1 $\pm$ 0.1	0	3.6 $\pm$ 1.2
M16	0.9 $\pm$ 0.3	12.5 $\pm$ 1.3	0.1 $\pm$ 0.04	1.9 $\pm$ 0.3	0	19 $\pm$ 5
Gyr-1207	ND	9.5 $\pm$ 2	ND	1.3 $\pm$ 0.2	ND	9 $\pm$ 6
MQ3A	0.4 $\pm$ 0.24	7.3 $\pm$ 0.8	0.06 $\pm$ 0.04	1.1 $\pm$ 0.3	0	4.6 $\pm$ 2.3

[a] See Table 3, footnote a.

the antimicrobial efficiency, no clear relationship can be found, either for the pulmonary bacterial reduction or for the presence or absence of emerging mutants. For a more comprehensive analysis, the bacterial contents in the lungs were split into nonmutated and mutated bacterial populations. Under these conditions, a clear relationship in the form of an E_{max} curve for the nonmutated population and a peak curve for the mutated population was observed for both antibiotics (Fig. 3).

Of note, the PK-PD$_{MIC}$ zone for the occurrence of mutants started at around the EC 90% of the E_{max} curve for the non-mutated bacterial population and were 52 and 11 for AUC/MIC and 5 and 1 for C_{max}/MIC for MFX and LVX, respectively. When the nonmutated population was considered, the efficacy of MFX was clearly higher than for LVX. Indeed, the E_{max} was only 4.4 log$_{10}$ CFU/g for LVX and 6.9 log$_{10}$ CFU/g for MFX.

(ii) PK-PD with MPC (PK-PD$_{MPC}$) and SI (PK-PD$_{SI}$). PK-PD parameters with MPC and SI are shown in Tables 4 and 5. The values of these parameters decreased with the increase in MPCs for both antibiotics. When there was a preexisting *parC* mutation, the PK-PD$_{MPC}$ values were not proportional to PK-PD$_{MIC}$ due to a sharp increase in the MPCs for these strains. In contrast, for the other strains, either susceptible (16089 and MS1A) or with a *gyrA* mutation (Gyr-1207) or a double *parC*-*gyrA* mutation (MQ3A), the MPCs were close to the MICs and so the PK-PD$_{MPC}$ parameters were close to those for PK-PD$_{MIC}$.

When the PK-PD parameters were calculated by using SI (PK-PD$_{SI}$), the obtained values for LVX and MFX were close to one another when the MICs and MPCs were also close, whether the strains were susceptible or resistant. When the MPCs were higher than the MICs, these PK-PD$_{SI}$ values decreased.

(iii) PK-PD with the MSW (PK-PD$_{MSW}$). T_{MSW} (expressed as a percentage) is the time during which the concentration in serum is between MIC and MPC; the AUC$_{MSW}$ is the area between the MIC and MPC values and the concentration-time curve. These values represent the extent of drug exposure within the MSW. The results are shown in Table 6. The observed values of T_{MSW} and AUC$_{MSW}$ increased as the difference between MICs and MPCs increased for both antibiotics (i.e., *parC* strains). Of note, the values for each drug were different. For example, the T_{MSW} ranged from 0 to 27% for LVX and from 0 to 96% for MFX.

Determination of the boundaries of the MSW for the LVX and MFX treatments. In order to delineate the zone associated with the occurrence of mutants with PK-PD parameters, a qualitative approach was done by using CART analysis.

The results are shown in Table 7. As can be seen, the results for the two antibiotics, regardless of the criteria considered, were very different. For example, the MSW, expressed as the free AUC/MIC ratio, was between 7.6 and 19.4 for LVX and between 50 and 164.5 for MFX.

The limits of the MSW were not significantly defined when PK-PD$_{MIC}$ parameters were used. However, significant results were observed for MFX when PK-PD$_{SI}$, or PK-PD$_{MSW}$ and, to a lesser extent, PK-PD$_{MPC}$ parameters were used. For example, when C_{max}/SI was considered, the MSW ranged from 0.1 to 0.2 for LVX ($P = 0.07$) and from 0.4 to 1 for MFX (p 0.02). When T_{MSW} was used to express the MSW, the percentages ranged from 2 to 25 for LVX ($P = 0.1$) and from 72.5 to 93.5 for MFX ($P = 0.02$).

DISCUSSION

Despite the currently low but increasing frequency of pneumococci with low-level resistance to fluoroquinolones, especially *parC* mutant strains, investigations on the efficacy of new fluoroquinolones on these organisms and patients infected with these infections is worthy of investigation.

In the present study with a rabbit model of human therapy, we showed that the occurrence of mutants is very high in animals when there is a preexisting *parC* mutation and that the

TABLE 5. Pharmacodynamic parameters with the SI[a]

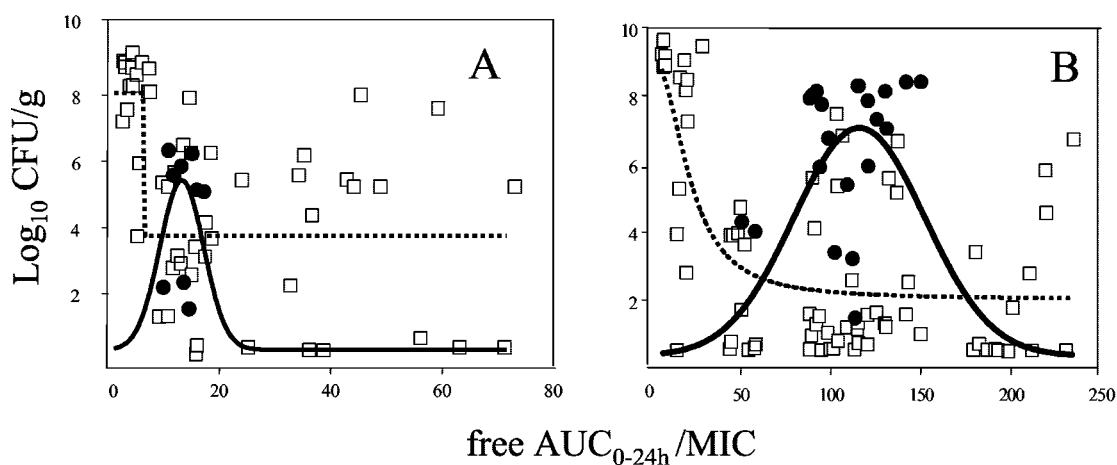| Strain | Mean value $\pm$ SD | | | |
| | AUC/SI | | C_{max}/SI | |
	LEV	MFX	LEV	MFX
16089	23 $\pm$ 7.8	25 $\pm$ 2†	2.9 $\pm$ 0.7	3.8 $\pm$ 0.8
MS1A	7.7 $\pm$ 3.3	29 $\pm$ 9	1.3 $\pm$ 0.6	4 $\pm$ 0.6
MS2A	1.2 $\pm$ 0.5	1.8 $\pm$ 0.2	0.1 $\pm$ 0.06	0.2 $\pm$ 0.08
MR3B4	1.6 $\pm$ 0.3	1.7 $\pm$ 0.3†	0.1 $\pm$ 0.05	0.2 $\pm$ 0.04
M16	7.5 $\pm$ 2.6	6.2 $\pm$ 0.6†	0.8 $\pm$ 0.3	0.9 $\pm$ 0.1†
Gyr-1207	ND	14 $\pm$ 3	ND	2 $\pm$ 0.3
MQ3A	6.8 $\pm$ 3.8	29 $\pm$ 3.5	1 $\pm$ 0.6	4.4 $\pm$ 1.2

[a] See Table 3, footnote a.

FIG. 3. Relationships of the free 24-h AUC/MIC obtained after treatment with LVX at 500 mg b.i.d. (A) or MFX at 400 mg o.d. (B) in a rabbit model of human therapy, with the residual bacterial pulmonary concentration ($\log_{10}$ CFU/gram) after 48 h of therapy. Symbols: $\square$, the nonmutated bacterial population; ●, represent the mutated bacterial population. The dose effect relationship is represented as an E_{max} curve for the nonmutated bacterial population (dashed line, $R^2 = 0.45$ and 0.45, $P < 0.05$, for LVX and MFX, respectively) and a peak curve for the mutated bacterial population (solid line, $R^2 = 0.72$ and 0.85, $P < 0.05$, for LVX and MFX, respectively).

values of the limits of the MSW are higher than the usual values of PK-PD criteria associated with the efficacy of fluoroquinolones (2, 3, 13, 14, 23, 40, 63, 74; O. Vesga and W. Craig, 36th ICAAC). Of note, all of these new in vivo mutants harbored a mutation in the gyrase gene, a finding which is concordant with in vitro data (6, 32, 33, 35, 46, 55).

In vitro, the mutation frequency of pneumococcal strains, with or without the presence of a *parC* mutation, is ca. 10^{-7} to 10^{-8} when the strains are exposed to fluoroquinolones (17, 31, 55). These observations contrast with the high rate of mutant emergence in our animal model, whatever the antibiotic studied. Indeed, after MFX treatment, mutations occurred in 88 to 100% of rabbits infected with a *parC79* mutant strain, whereas only 18% of rabbits infected with the *parC83* mutated strain exhibited resistant mutants. The mutation rate observed in this in vivo study seems to be higher than that reported in the in vitro PK-PD models (40, 46, 75).

This high in vivo mutation rate is likely to be explained by the high pneumococcal concentration in the lungs in our model (ca. 8 $\log_{10}$ CFU/g). These concentrations allow detection of emerging mutants since the mutation frequency is in the range

of 10^{-7} to 10^{-8}. Importantly, these bacterial concentrations are of the same order of magnitude as those observed in acute exacerbation of chronic bronchitis (50) and less than those observed in pneumococcal human lobar pneumonia (26, 38). Other factors, such as the duration of antibiotic exposure (48 h), sub-MIC concentrations, antibiotic tissue fixation, and ischemic pulmonary phenomena, can interfere with the efficacy of these molecules.

Moreover, the occurrence of resistant mutants in vivo could be anticipated based on the MPC results. Indeed, the MPCs of *parC* mutant strains were very high. These MPCs are far above the C_{max} obtained during standard LVX regimens in humans and close to the C_{max} for MFX. These concentrations in humans were reproduced in our rabbit model and were higher than the MICs for the mutated strains but not for the resistant strains, and mutations occurred for those strains with high MPC values (i.e., with a preexisting *parC* mutation), in other words, when the MSW was wide (measured as a high value of SI, from 4 to 32, depending on the strains and the antibiotic used [see Table 1]). For strains with a low SI, no mutants were recovered in treated rabbits, either because these strains were

TABLE 7. Determination of in vivo boundaries of MSW by using CART analysis from PK-PD results obtained in rabbits infected with pneumococci exhibiting various degrees of susceptibility to fluoroquinolones and treated with LVX (500 mg b.i.d.) or MXF (400 mg o.d.)

| Parameter | MSW | | | | | |
| | LVX | | | MXF | | |
	Lower bound	Superior bound	P	Lower bound	Superior bound	P
AUC/MIC	7.6	19.4	0.13	50.0	164.5	0.12
C_{max}/MIC	0.8	1.8	0.07	7.3	21.5	0.21
T>MIC	3.5	25.5	0.13	98		0.28
AUC/MPC	0.5	1	0.17	5.9	14.5	0.16
C_{max}/MPC	0.05	0.1	0.05	0.9	1.9	0.10
T>MPC					26	0.1
AUC/SI	1.1	2.25	0.11	2.2	7.3	0.04
C_{max}/SI	0.1	0.2	0.07	0.4	1	0.02
AUC_{MSW}	0.55		0.21	14.3	27.6	0.04
T_{MSW}	2	25	0.10	72.5	93.5	0.02

resistant or because the MPCs were very close or equal to the MICs.

Although the MSW is less well delineated for LVX (perhaps due to the limited activity of this antibiotic), our results confirm the concept of the MSW since the PK-PD$_{MPC}$, PK-PD$_{SI}$, and PK-PD$_{MSW}$ parameters are associated with the occurrence of mutants in animals infected with *parC* strains, whereas the PK-PD$_{MIC}$ parameters are not (Table 7). Indeed, the MSW defined by using the MICs is very large and begins at AUC/MIC ratios that are much higher than the 30 to 50 values usually considered as predictive for the efficacy of fluoroquinolones against pneumococcal infections (3, 13, 22, 40, 74; D. Croisier, D., C. Lequeu, C. Neuwirth, L. Piroth, M. Froidure, H. Portier, and P. Chavanet, 42nd ICAAC, abstr. B-708, 2002; O. Vesga and W. Craig, 36th ICAAC); as suggested, the AUC/MIC ratio is not adequate to predict the emergence of resistant mutants (45). For example, in our model, resistant mutants were recovered for AUC/MICs from 50 to 164.5 with MFX. These findings are completely concordant with the in vitro theory of Zhao et al. (76, 77). Indeed, in our in vivo model, we also observed that, as the antibiotic exposure increases, pulmonary concentrations of susceptible pneumococci first decreased, and then emerging mutants were recovered (Fig. 2). In these conditions, it is not surprising that PK-PD parameters with MPCs, SI, or MSW are more adequate to delineate the MSW (Tables 4 to 6).

Although only the free fraction of antibiotics was used for calculations, it is noteworthy that values of these PK-PD parameters and the MSW bounds are very different for LVX and MFX. This observation can be related to the higher intrinsic antibacterial activity of MFX, as shown by relatively low MIC and MPC values compared to the values of LVX. Furthermore, these differences of in vitro intrinsic activities are also well translated in vivo; indeed, when the bacterial population without emerging mutants was considered, MFX was more active than LVX. Considering these results together and recognizing that only two molecules have been tested to date, we suggest that it will likely be difficult to establish a universal PK-PD parameter for MSW delineation for antibiotics with different intrinsic activities.

Recently, reports of failures to LVX treatment of pneumococcal diseases have been occurring (16, 23, 25, 37, 39, 64, 69, 71). Some of these failures occurred in patients previously exposed to fluoroquinolones and/or when the pneumococcus exhibited an decreased susceptibility to fluoroquinolones, which was sometimes related to a preexisting *parC* mutation. In our experimental study, we reproduced both situations: mutation and a failure to reduce the pulmonary pneumococcal concentration. It is generally considered that a bacterial reduction of 2 $\log_{10}$ CFU/g is associated with antibacterial efficacy. According to these criteria, MFX remained highly active for pneumonia due to wild-type strain and the strain exhibiting efflux (Fig. 2). However, when there was a preexisting *parC* mutation, both LVX and MFX treatments were associated with both emerging mutations and a failure to reduce the bacterial pulmonary concentrations (Fig. 1 and 2). These experimental observations are completely concordant with those from clinical situations (29). Considering the current low rate of pneumococci with low-level resistance to fluoroquinolones, a clinical trial to assess the efficacy of these drugs on pneumo-

TABLE 6. Pharmacodynamic parameters with the MSW[a]

Strain	Mean value ± SD			
	AUC$_{MSW}$		T_{MSW}	
	LVX	MFX	LVX	MFX
16089	0	0†	0	0†
MS1A	15 ± 8	3.1 ± 6	27 ± 14	17.2 ± 34
MS2A	11 ± 13	25 ± 2.6	14.8 ± 15	96 ± 1
MR3B4	12 ± 7.5	25 ± 4.2	13.8 ± 7.8	96.3 ± 1.2
M16	0	15 ± 1.3	0	77.9 ± 8
Gyr-1207	ND	12 ± 4	ND	34.7 ± 10.8
MQ3A	1.2 ± 2.7	0†	0.4 ± 0.8	0†

[a] See Table 3, footnote *a*.

nia due to such strains is probably unrealistic. Consequently, we think that these experimental investigations in a rabbit model of human therapy, one used for studying difficult clinical situations (i.e., strains with low level of resistance), have to be done in order to determine the clinical situations for which the use of quinolones should or should not be supported.

From our in vivo experimental results based on a rabbit model of human therapy, we conclude that, despite the higher intrinsic efficacy of MFX on pneumococcal pneumonia compared to LVX, the rate of emerging mutation is high and the same for both drugs when a low-level fluoroquinolone resistance due to *parC* mutations is preexisting. These strains exhibit high MPCs and are associated with the occurrence of mutants when the drug concentrations in serum are between MICs and MPCs (that is, in the MSW), which confirms the in vitro concept. However, the MPC methodology is currently too time-consuming to be done routinely. Thus, because of the limited interest of the MIC tests for the detection of *parC* mutant strains (4, 49), other rapid tests are needed for the detection of these particular pneumococcal strains to confirm or complement the clinical suspicion of infections due to such strains in order to avoid pneumococcal mutations and/or clinical failures (65). Experimental investigations can bring arguments to approximate PK-PD parameters (using MPCs in addition to MICs) associated with the absence of mutation.

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