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Efficacy and pharmacodynamics of simulated human-like treatment with levofloxacin on experimental pneumonia induced with penicillin-resistant pneumococci with various susceptibilities to fluoroquinolones

Delphine Croisier, Pascal Chavanet*, Catherine Lequeu, Abderrahmane Ahanou, Anne Nierlich, Catherine Neuwirth, Lionel Piroth, Michel Duong, Marielle Buisson and Henri Portier

Service des Maladies Infectieuses, Microbiologie Médicale et Moléculaire (EA562), Hôpital du Bocage, BP 1542, 21034 Dijon Cedex, France

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Newer fluoroquinolones, such as levofloxacin, have shown an enhanced *in vitro* and *in vivo* activity against penicillin-resistant *Streptococcus pneumoniae* infections. The frequency of *S. pneumoniae* with reduced susceptibility to quinolones, although currently low, raises the question of the therapeutic efficacy of levofloxacin on infection due to such strains. We used an animal model of penicillin-resistant pneumococcal pneumonia using six strains with various levels of susceptibility to ciprofloxacin and levofloxacin in rabbits to induce pneumonia, and simulated a human-like treatment of 500 mg twice a day for 48 h. Strains' susceptibility profiles for ciprofloxacin and levofloxaxin were (ciprofloxacin/levofloxacin MIC, mg/L; genotype): 0.5/0.5 (Cip0.5), 2/1 (Cip2), 4/1.75 (Cip4), 8/1.75 (*parC* mutation) (Cip8), 10/2 (*parC* mutation) (Cip10), 64/16 (*parC* and *gyrA* mutations) (Cip64), respectively. All the strains induced a crude pneumonia in all rabbits. Significant bacterial reductions at the end of treatment in lung and spleen were observed for the four former strains ($P < 0.05$) but not for the latter two. An AUC/MIC ratio of at least 32 identified 95% of an at least bacteriostatic effect ($P = 0.038$) and 76% of a bactericidal effect ($P = 0.09$). Mutants were detected in treated animals infected with strains harbouring *parC* mutations (Cip8 and Cip10) and when the AUC/MIC ratio was between 13 and 31. We conclude that levofloxacin is effective against experimental pneumonia due to pneumococci with MIC < 1.5 mg/L, ineffective on experimental pneumonia due to pneumococci with MIC ≥ 2 mg/L, and could be associated with the appearance of mutants when a *parC* mutation is pre-existing.

Introduction

Pneumonia remains a leading cause of death throughout the world and *Streptococcus pneumoniae* is the most common cause leading to significant morbidity and mortality.[1–4] The prevalence of antibiotic-resistant *S. pneumoniae* has increased over the last decade and β-lactam resistance is associated with increased morbidity and mortality.[2,5,6]

In this context, alternative treatments for pneumococcal pneumonia are needed. Among these alternatives, new fluoroquinolones with extended activity against Gram-positive aerobes including pneumococci could be proposed. Levofloxacin has been licensed for this indication in many countries based on several clinical trials that provided favourable results.[7–9] In these studies, all of the isolated pneumococci were fully susceptible to fluoroquinolones.

Several reports described pneumococci with reduced susceptibility to ciprofloxacin and other quinolone molecules,[10–17] raising the question of the efficacy of levofloxacin for infections due to pneumococci susceptible to levofloxacin but with various susceptibility levels to ciprofloxacin.

The aim of this study was to investigate the efficacy of a simulated human-like treatment, i.e. levofloxacin intravenously (iv) 500 mg twice a day (the recommended regimen for

*Corresponding author. Tel: +33-3-8029-3637; Fax: +33-3-8029-3638; E-mail: p.chavanet@planetb.fr

severe pneumonia in France), on experimental pneumonia in rabbits induced by penicillin-resistant pneumococci with various degrees of susceptibility to ciprofloxacin and levofloxacin.

Materials and methods

Bacterial strain and growth conditions

One *S. pneumoniae* strain isolated from the blood of a patient with pneumonia was used [kindly provided by the Centre National de Référence des Pneumocoques (Dr Geslin), Créteil, France]. The strain (strain Cip0.5, serotype 9V) was highly resistant to penicillin (MIC = 4 mg/L). Purity was confirmed throughout the study by Gram staining and purity plating. Working stock cultures were kept frozen at –70°C in a 15% glycerol-supplemented brain–heart infusion (BHI) broth (bioMérieux Laboratories, Marcy l'Étoile, France). In order to maintain virulence, stock cultures were replaced every month using the colonies isolated from rabbits with *S. pneumoniae* untreated pneumonia.

Isolation of quinolone-resistant mutants

Mutants were obtained *in vitro* as described previously.[18] Briefly, a heavy culture of strain Cip0.5 was resuspended and spread onto Mueller–Hinton agar plates supplemented with 4% horse blood (bioMérieux) containing ciprofloxacin (1 ×, 2 × or 4 × MIC) and incubated at 35°C for 3 days. Colonies appearing after 2 or 3 days were subcultured onto agar plates containing the same concentration as the initial plates.

MIC determination

MICs were determined by the standard method in agar.[19] Inocula of 5×10^5 cfu were spotted onto Mueller–Hinton agar plates supplemented with 4% horse blood and containing ciprofloxacin (Bayer Pharma, Puteaux, France) or levofloxacin (Roussel-Uclaf, Romainville, France). MICs were read after 18 h of incubation at 37°C. The ciprofloxacin and levofloxacin susceptibility of the obtained strains was also determined by a broth MIC method.[19,20]

Time–kill curves

The *in vitro* bactericidal activity of levofloxacin on the different strains was evaluated as described previously.[21] The bacterial growth in test and control tubes was counted at 0, 3, 6, 12 and 24 h after incubation at 37°C. The initial inoculum size was 5×10^5 cfu/mL. The concentration of levofloxacin was 6 mg/L, corresponding approximately to the maximal blood concentration observed in humans.

Evaluation of the efflux

MIC determinations were done in parallel in the presence or absence of an efflux inhibitor (10 μg of reserpine/mL).[22–24] The growth inhibition assay was also used[25] with slight modifications; briefly, 1×10^6–2×10^6 cfu/mL were inoculated into BHI medium (Biomérieux) containing one-fourth the MIC and in the presence or absence of 10 μg of reserpine/mL; measurements were determined over 7 h of incubation at 35°C. The extent of growth inhibition was determined by comparing the areas under the optical density (OD) curve (550 nm) of the cultures with those of the controls (percentage decrease in OD).

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 the *parC*, *parE*, *gyrA* and *gyrB* genes as previously described.[26] PCR primers (Genset, Paris, France) were those previously reported.[27] Conditions for PCR and for asymmetric PCR (to provide single-stranded DNA) prior to DNA sequencing (Génome Express S.A., Grenoble, France) were as described previously.[26,28,29]

Preparation of the inoculum

Before each animal experiment, several *S. pneumoniae* strains from one aliquot (per strain) were inoculated into BHI broth, cultured on agar plates and incubated for 24 h at 37°C in 5% CO_2. Colonies (25–30) were taken and inoculated into 9 mL of BHI 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 physiological saline in order to obtain a final concentration of 10 $\log_{10}$ cfu/mL. No adjuvant was used. These concentrations were first determined by using OD measurements, with reference to a standard curve, and confirmed by using successive dilution cultures.

Animals

Male New Zealand white rabbits (body weight 2.5–2.7 kg) were obtained from Elevage Scientifique des Dombes (Romans, France). These animals were not immunosuppressed and had a sanitary status of virus antibody free and specific pathogen free. They were placed in individual cages and were nourished *ad libitum* with drinking water and feed, according to current recommendations.

Experimental pneumococcal pneumonia in rabbits

The animals were anaesthetized intramuscularly with 1.5–2 mL of a mixture of ketamine (500 mg/mL) and xylazine (2.75 mg/mL). Two silicone catheters were introduced into

the jugular vein (a short one with extremity in the superior vena cava,[30] and a longer one with extremity in the right auricula) through a lateral incision of the neck, and then subcutaneously tunnelled through the interscapular area. The short catheter was introduced in order to infuse antibiotics at human pharmacokinetic rates and the other was placed to draw blood samples at timed intervals. Twenty-four hours later, the rabbits were anaesthetized intravenously by using 0.6–0.8 mL of the ketamine-plus-xylazine mixture and then by a few millilitres of propofol as needed. Under view control, a silicone catheter (Sigma Medical, Nanterre, France) was introduced through the vocal cords into the trachea and pushed until it reached the bronchia. Freshly prepared pneumococcal inoculum (0.5 mL) was then gently flushed through this catheter. The endobronchial catheter was then immediately removed after the inoculum instillation, and the animals were placed upright for 15 s to facilitate distal alveolar migration by gravity.

Experimental pneumonia examination

Macroscopic criteria. For each strain, experimental pneumonia was evaluated as previously described.[31] Briefly, postmortem examination was performed after anaesthesia by using overdoses of thiopental. For each rabbit, the thorax was opened, and the existence of pleural effusion was noted. The lungs were then dissected aseptically, and put on a sterile gauze for at least 5 min, to allow residual pulmonary blood absorption. A laparotomy was then performed, and the spleen was aseptically removed. An overall macroscopic score was calculated as the sum of all lobar macroscopic scores, plus 2 points in the case of pleural effusion (range 0–39 points).[31,32]

Pulmonary oedema measurement. The global pulmonary permeability, which is a key factor in pneumonia, especially due to pneumococci, was measured as described previously.[33] Briefly, each pulmonary lobe was weighed and homogenized in sterile water and an aliquot was weighed and evaporated at 40°C for 4 days to constant weight. The ratio wet weight/dry weight is the expression of the intensity of the pulmonary oedema.

Bacterial content in lungs and spleen. Each pulmonary lobe was weighed and homogenized in sterile serum saline. The spleen was prepared under the same conditions. Bacteria were counted in a sample of this crude homogenate by plating 10-fold dilutions on sheep blood agar and incubating the plates for 24–48 h at 37°C. Bacterial concentrations in each lobe or in the spleen were determined after adjusting for weight. The threshold value was 1 $\log_{10}$ cfu/mL (for low bacterial concentrations 1 mL was plated). For statistical comparisons of the difference between densities of bacteria in the lungs, culture-negative lobes were considered to contain 1 $\log_{10}$ cfu/g. For each rabbit, the mean pneumococcal pulmonary concentration was calculated according to each lobar bacterial concentration with lobar weight [e.g. mean concentration = Σ(lobar concentration $\times$ lobar weight)/Σ lobar weights].

In vivo mutants. In the treated animals and for each lobe or spleen with residual surviving bacteria, mutants were isolated by plating 1 mL of the crude tissue homogenate and 10-fold dilutions on sheep blood agar containing 2 $\times$ and 4 $\times$ MIC for the strain under test.

Simulation of human levofloxacin pharmacokinetics in rabbits

Levofloxacin assay. The drug was reconstituted from laboratory powder of known potency according to the manufacturer's instructions, just before each experiment. Concentrations in blood were determined by the disc plate bioassay method.[34] The bioassay microorganism was *Escherichia coli* NIJJHC2, the growth medium was antibiotic medium no. 2 (Difco Laboratories, Detroit, MI, USA). Standard curves were established with solutions of levofloxacin (progression from 0.5 to 7 mg/L) in sterile water. The linearity of the standard curves used for disc plate bioassays was at least 0.98 (r^2). The concentrations in serum were derived from the standard curves. The serum samples were diluted in sterile 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 5.5% and 7.0%, respectively.

Pharmacokinetic analysis after one short infusion of levofloxacin. A 10 mg/kg solution of levofloxacin (Tavanic; Aventis, Paris la Défense, France) was infused over 30 min in 10 rabbits and blood punctures were performed at 0, 5, 10, 15, 20, 30, 45, 60, 90 and 120 min after injection. Sera from these blood samples were stored at –70°C until assay. Standard kinetic parameters were determined.[35,36] The area under the serum concentration–time curves (AUC) was calculated using the trapezoidal rule and included all experimental data points. A compartmental analysis was done using Kinetica software (Innaphase, Champs sur Marne, France). The analysis was based on the Akaike criteria.[37]

Human-like treatment. The objectives were to simulate the human pharmacokinetics that follow the administration of levofloxacin 500 mg iv twice a day[38] for 48 h. The procedure to compensate for the faster elimination of antibiotic in small animals compared with humans was described previously.[31] Briefly, from the pharmacokinetic parameters of levofloxacin, the timed interval compensatory dose can be calculated to

obtain the desired (human) concentrations.[39,40] A variable flow rate infusion with successive levels was used. For each experiment, a computer-controlled pump, for administration of levofloxacin, was connected to a central venous catheter. This protected connection allowed free circulation and free food access for the rabbits. Infusion rates were controlled by programmable computer software (Softpump; World Precision Instruments, Sarasota, FL, USA).

Individual pharmacokinetics. In order to check that the administered treatment resulted in a simulation of human treatment, blood samples were obtained for each treated rabbit through the second central venous catheter. Levofloxacin concentrations were measured as described above. From these blood concentrations, the best PK fit was determined (Akaike criteria) and then, for each treated animal, a simulation of the antibiotic pharmacokinetics was obtained using Kinetica software.

Therapeutic and analysis timing

The simulated human-like treatment began 4–5 h (start) after the bronchial inoculation. The treatment lasted 48 h, corresponding to the equivalent of levofloxacin 500 mg over 1 h in humans at T_0 and then 12, 24 and 36 h. Post-mortem examinations were carried out a few hours after the 48th hour in order to avoid any carry-over effect. At that time, no residual antibiotic concentration was experimentally detected in the serum.

Pharmacodynamic analysis

From individual pharmacokinetics of each treated animal, the following pharmacodynamic (PD) parameters were calculated versus each strain's susceptibility (MIC): peak of concentration/MIC (C_{max}/MIC), AUC/MIC and time of concentration above MIC ($T > $MIC).

Statistical analysis

The results were expressed as the mean or percentage ± S.E.M. Differences between quantitative values were analysed by using the Mann–Whitney non-parametric test. Continuous variables were analysed with one-way analysis of variance. In the case of a significant test, *post hoc* analysis comparing results for each treated arm versus each untreated arm was conducted by using Dunnett's test. To analyse relationships between quantitative values, a correlation coefficient r^2 value was calculated by the linear regression model. Several categorizations were established:

(i) The strains were classified as follows: (a) with or without significant efflux of ciprofloxacin (at least two-fold reduction of MIC); (b) three genotypic profiles: wild-type (wt), the presence of a *parC* mutation (*parC*) (strains Cip8 and Cip10) and the presence of both *parC* and *gyrA* mutations (strain Cip64).

(ii) The antibacterial effect was counted as the difference of cfu/g of lung between controls and treated animals for each tested strain allowing the following classification: a bacteriostatic effect was defined as between no reduction in bacterial count and $<2 \log_{10}$ cfu/g reduction, and a bactericidal effect as $\geq 2 \log_{10}$ cfu/g reduction.

(iii) At the end of treatment, the animals were classified according to the presence or absence of mutants in the lungs.

Several logistic regressions were performed: the dependant variables were a bacteriostatic or bactericidal effect or the presence or absence of *in vivo* mutants, the independent variables were the PK–PD parameters, the presence or absence of efflux of ciprofloxacin and the genotypic profiles; they were entered into the model of the multivariate analysis if $P < 0.2$ was reached in the univariate analysis. Since the three PK–PD parameters were highly correlated, AUC/MIC was chosen as the independent variable. A clustering analysis (classification tree technique) was also performed to investigate the treatment effects in subgroups by entering the same independent and dependent variables.[41,42] For all the tests, a P value <0.05 was considered significant. All calculations were done with SPSS software (SPSS Inc., Chicago, IL, USA).

Results

Antimicrobial susceptibility of the obtained S. pneumoniae

The six *S. pneumoniae* strains with various levels of fluoroquinolone susceptibility obtained after successive passages on ciprofloxacin are described in Table 1. All these strains, except strain Cip64, were susceptible to levofloxacin (≤2 mg/L). The Cip0.5 strain was fully susceptible to ciprofloxacin (MIC = 0.5 mg/L), the strain Cip2 had intermediate susceptibility and the other strains had MICs from 4 to 10 mg/L; the MIC for strain Cip64 was 64 mg/L of ciprofloxacin and 16 mg/L of levofloxacin; this latter strain was included as a negative control.

The effect of reserpine on fluoroquinolone susceptibility was weaker for levofloxacin than for ciprofloxacin. In terms of MIC, there was no effect of reserpine on the susceptibility to ciprofloxacin for strains Cip0.5 and Cip2; however, the inhibition growth test of reserpine showed marked differences. The Cip0.5 strain, although of wild-type, exhibited a marked efflux of ciprofloxacin[43] but none of levofloxacin. The other strains exhibited efflux of ciprofloxacin. As shown in Table 1, there was no clear relationship between the susceptibility level and both the intensity of efflux and the presence of one mutation in *parC*. Finally, the presence of reserpine induced at least a two-fold reduction of the MIC of ciprofloxacin for strains Cip4, Cip8, Cip10 and Cip64; therefore,

Table 1. Ciprofloxacin and levofloxaxin susceptibility: MICs, percentage of bacterial growth inhibition in the presence of reserpine, and genotypic mutations

Strains	MIC (mg/L)				Efflux (% reduction)[a]		Genotype and mutation			
	ciprofloxacin		levofloxacin							
	without reserpine	with reserpine	without reserpine	with reserpine	ciprofloxacin	levofloxacin	*parC*	*parE*	*gyrA*	*gyrB*
Cip0.5	0.5	0.5	0.5	0.5	51	0	–	–	–	–
Cip2	2	1	1	0.75	52	31	–	–		–
Cip4	4	0.5	1.75	0.75	24	16	–	–	–	–
Cip8	8	2	1.75	1	39	0	Ser^{79}→Phe	–	–	–
Cip10	10	2	2	1	34	14	Ser^{79}→Phe	–	–	–
Cip64	64	16	16	10	43	15	Ser^{79}→Tyr	–	Ser^{81}→Phe	–

[a]See Materials and methods.

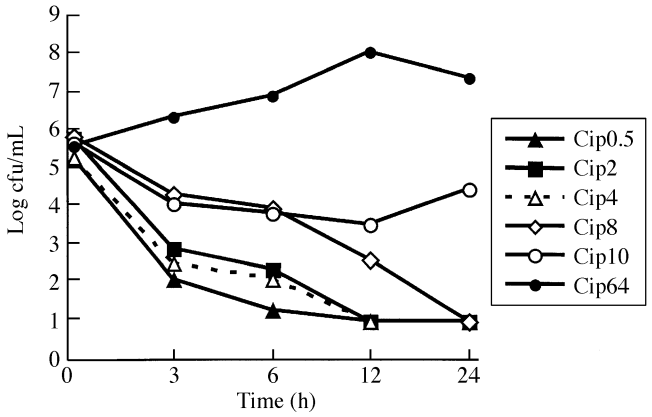

Figure 1. *In vitro* killing curves of levofloxacin (6 mg/L) on pneumococci with various susceptibility levels to fluoroquinolone (means of three experiments in duplicate). MICs of ciprofloxacin and levofloxacin were, respectively: strain Cip0.5, 0.5 and 0.5 mg/L; strain Cip2, 2 and 1 mg/L; strain Cip4, 4 and 1.75 mg/L; strain Cip8, 8 and 1.75 mg/L; strain Cip10, 10 and 2 mg/L; strain Cip64, 64 and 16 mg/L.

these strains were classified as possessing a significant efflux mechanism.

The *in vitro* bacterial killing of levofloxacin at 6 mg/L is shown in Figure 1 (mean of three experiments performed in duplicate). The killing was complete for the strains Cip0.5 and Cip2. Although the two strains Cip4 and Cip8 exhibited the same MIC (1.75 mg/L), the *in vitro* killing was delayed for the strain Cip8; this latter strain had a weak efflux of levofloxacin and a *parC* mutation. For the strain Cip10 (MIC = 2 mg/L), a regrowth was observed.

Simulated human levofloxaxin treatment

In healthy rabbits, levofloxacin concentrations in serum following a short infusion of 10 mg/kg over 30 min fitted with a two-compartment model with the following constants: $A = 15$ mg/L, $\alpha = 6\ h^{-1}$, $B = 4.5$ mg/L and $\beta = 0.5\ h^{-1}$. Then, by using these parameters and the required compensation, as described in Materials and methods, the simulation of the expected levofloxacin concentrations was achieved in rabbits. Figure 2 shows that this curve superimposed almost exactly the human curve of serum concentrations observed after 500 mg iv infused over 1 h. The observed AUC (from 0 to 12 h) was 27 ± 8 mg·h/L ($n = 6$). Therefore, this infusion procedure was performed for each infected and treated rabbit and adapted for each weight of animal. After the first 12 h infusion, the following infusions took into account the residual (trough) concentration.

In each treated animal, at least six blood samples were drawn for levofloxacin assay; the best PK fit was determined and then an individual PK simulation was done. The results are shown in Table 2 (groups of animals are noted by strain). The peaks of levofloxacin concentration were in the range of

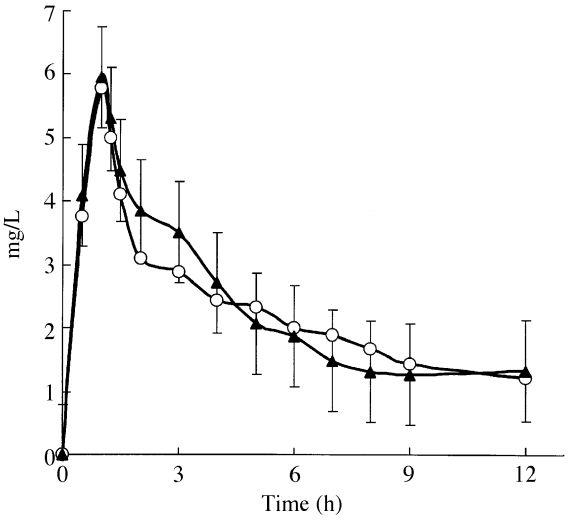

Figure 2. Human-like pharmacokinetics of levofloxacin in rabbit (equivalent in man to 500 mg iv over a 1 h infusion). Desired human concentrations (circles); obtained concentrations in rabbits (triangles).

those observed in humans. The trough levels of levofloxacin were also in the range of human ones and tended to increase over time. Overall, the global levofloxacin exposure (AUC) was similar to that observed in humans and was similar between groups.

Effects of the treatment on the experimental pneumococcal pneumonia

Pulmonary pathological score and oedema (Table 3). All the pneumococcal strains were associated with the same early (5th hour) and late (end of treatment) lesional score in untreated animals. Furthermore, this macroscopic score was also not different in treated and untreated rabbits at the end of the experiment.

In untreated animals, all the strains were associated with the same order of pulmonary oedema. Levofloxacin treatment was associated with a protective effect on the pulmonary permeability only when rabbits were infected with the most susceptible pneumococcal strain (strain Cip0.5) ($P = 0.008$).

Antimicrobial effect of levofloxacin-simulated human-like treatment (Figure 3). In untreated animals, the microbial concentrations were not different whatever the strain used for the experimental pneumonia both in lung and spleen. A significant reduction of bacterial content in lung was induced by the simulated human-like treatment with levofloxacin for all the strains, but not with the strain Cip10, which has an MIC of 2 mg/L of levofloxacin (Figure 3a). As anticipated, no antibacterial effect was observed for the levofloxacin-resistant strain Cip64. Similar observations were made for pneumococcal content in spleen; however, the bacterial reduction in spleen for the Cip2 group was not statistically significant as compared with controls without treatment (Figure 3b).

Mutants. Mutants were found in pulmonary lobes of two of 10 treated rabbits infected with the strain Cip8. The pulmonary concentration of these mutants was 5.3 $\log_{10}$ cfu/g. No mutants were found in the spleen of these two animals. For seven of the 10 treated animals infected with the strain Cip10, mutants were detected in the lungs ($5.1 \pm 1.9 \log_{10}$ cfu/g) but in only one spleen. The MIC of these mutants was 16 mg/L of levofloxacin. These mutants acquired a *gyrA* mutation. No mutants were found in rabbits infected with the other strains (including strain Cip64).

Pharmacodynamics

PK–PD and bacterial clearance. The results for the three PK–PD parameters, AUC/MIC, C_{max}/MIC and time of concentration above MIC ($\%T >$ MIC) obtained from the data of each treated animal are shown in Table 4. As expected, these parameter values decreased as the MIC increased. These indices were strongly correlated: AUC/MIC and C_{max}/MIC,

Table 2. Pharmacokinetics of simulated human-like treatment with levofloxacin (equivalent to 500 mg iv twice a day for 48 h) in infected and treated rabbits

			Concentration (mg/L) at				
				trough (h)			
Group of treated animals by strain	*n*	AUC_{0-24} (mg·h/L)	peak	12	24	36	48
Cip0.5	9	41 ± 5	5.1 ± 1.4	0.7 ± 0.3	1.3 ± 0.5	1.4 ± 0.6	1.4 ± 0.6
Cip2	8	43 ± 7	5.5 ± 3	0.6 ± 0.2	1.1 ± 0.5	1.1 ± 0.5	1.1 ± 0.6
Cip4	8	49 ± 7	6.7 ± 2.2	0.7 ± 0.5	1.3 ± 0.7	1.3 ± 0.7	1.3 ± 0.8
Cip8	9	56 ± 8	7.3 ± 4	0.97 ± 0.5	1.6 ± 0.8	1.7 ± 0.8	1.8 ± 0.8
Cip10	11	43 ± 4	5.2 ± 2	0.7 ± 0.2	1.2 ± 0.3	1.2 ± 0.3	1.25 ± 0.3
Cip64	9	49 ± 7	6.9 ± 4	0.7 ± 0.3	1.3 ± 0.5	1.3 ± 0.5	1.4 ± 0.6

Table 3. Pulmonary macroscopic score (macro.) and permeability (permeab.) (see Materials and methods) in the experimental pneumonia due to pneumococci with various susceptibility levels to fluoroquinolone in rabbits untreated or treated with a 48 h levofloxacin human-like treatment

	Time of sampling					
	controls without treatment				levofloxacin treatment	
	start		sacrifice		sacrifice	
Group of treated animals by strain	macro.	permeab.	macro.	permeab.	macro.	permeab.
Cip0.5	12 ± 7	–	24 ± 10	5.6 ± 0.6	20 ± 6	4.8 ± 0.4[a]
Cip2	22 ± 8	–	22 ± 7	5 ± 2	19 ± 3	5.4 ± 0.5
Cip4	17 ± 8	–	30 ± 4	5.9 ± 2	25 ± 8	5 ± 0.5
Cip8	15 ± 8	–	26 ± 5	5.3 ± 1	25 ± 5	5.4 ± 0.7
Cip10	21 ± 5	–	24 ± 6	5.3 ± 1.6	24 ± 4	5.2 ± 0.3
Cip64	14 ± 5	–	21 ± 8	6.6 ± 3	22 ± 10	5.8 ± 0.8

[a]Significantly different compared with controls ($P = 0.008$).

$r^2 = 0.9$, $P < 0.0001$; $T >$ MIC was less associated to the two other indices, $r^2 = 0.8$ and $r^2 = 0.75$, $P < 0.01$.

The association between these PK–PD parameters and the bacterial reduction in lungs was tested. A very weak association was found with $T >$ MIC ($r^2 = 0.09$, $P = 0.02$). There was also an association between the bacterial reduction in lung and AUC/MIC ($r^2 = 0.24$, $P = 0.0001$) and C_{max}/MIC ($r^2 = 0.19$, $P = 0.0007$).

Several univariate logistic regressions were done with these PK–PD parameters as independent variables and two different levels of bacterial effect as dependent variable. When a bacteriostatic effect was tested, only AUC/MIC and C_{max}/MIC remained significant ($\chi^2 = 5.8$, $P = 0.01$ and $\chi^2 = 6.4$, $P = 0.01$, respectively). For a ≥2-log killing effect, no AUC/MIC or C_{max}/MIC ratios remained significant ($P > 0.15$); $T >$ MIC raised significance ($\chi^2 = 3.3$, $P = 0.06$). Since these three PK–PD parameters are strongly correlated, the multivariate analysis was not done.

Furthermore, a significant logistic association was found between a bacteriostatic effect and the genotypic profiles of the strains ($\chi^2 = 6$, $P = 0.04$) and the presence or absence of efflux of ciprofloxacin ($\chi^2 = 10.5$, $P = 0.0012$). A multivariate analysis was done by introducing these two latter variables and AUC/MIC; only the efflux profile remained significant ($\chi^2 = 4.69$, $P = 0.03$). When the bactericidal effect was considered, only the genotypic and efflux profiles were significant in univariate analysis ($\chi^2 = 7.4$, $P = 0.02$ and $\chi^2 = 3.4$, $P = 0.06$, respectively); none of these variables remained significant in the multivariate analysis.

Since these latter analyses were not really contributive, a categorical analysis was performed. An AUC/MIC ratio of at least 32 identified 95% of an at least bacteriostatic effect ($P = 0.038$) and 76% of a bactericidal effect ($P = 0.09$). Furthermore, when AUC/MIC ratio, the genotypic and efflux profiles were entered in the model, a bacteriostatic effect was significantly associated with the presence or absence of efflux of ciprofloxacin then to the genotypic profile and then to an AUC/MIC ratio of 26 ($P = 0.02$).

PK–PD and bacterial mutations. The PK–PD parameters of the animals in which mutants were detected were: AUC/MIC = 22 ± 5.4, C_{max}/MIC = 2.5 ± 0.7, and $T >$ MIC = 13 ± 7.6.

A categorical approach identified all the mutants for an AUC/MIC ratio between 13 and 31 ($P = 0.1$), for a C_{max}/MIC ratio below 3.5 ($P = 0.03$) and for $T >$ MIC below 25 ($P = 0.1$).

It is noteworthy that all the mutants were observed in rabbits infected with strains possessing both efflux of ciprofloxacin and a *parC* mutation.

Discussion

In this study, we explored the *in vivo* efficacy of levofloxacin (by simulating a human-like treatment equivalent to 500 mg iv twice a day) on experimental pneumonia in immunocompetent rabbits due to penicillin-resistant pneumococci with various susceptibilities to ciprofloxacin but susceptibility to levofloxacin (one strain was resistant to levofloxacin as a negative control). We found that this treatment was inefficient not only, as expected, when a fully resistant strain was used, but more surprisingly, when borderline susceptible strains were used with secondary appearance of levofloxacin-resistant mutants.

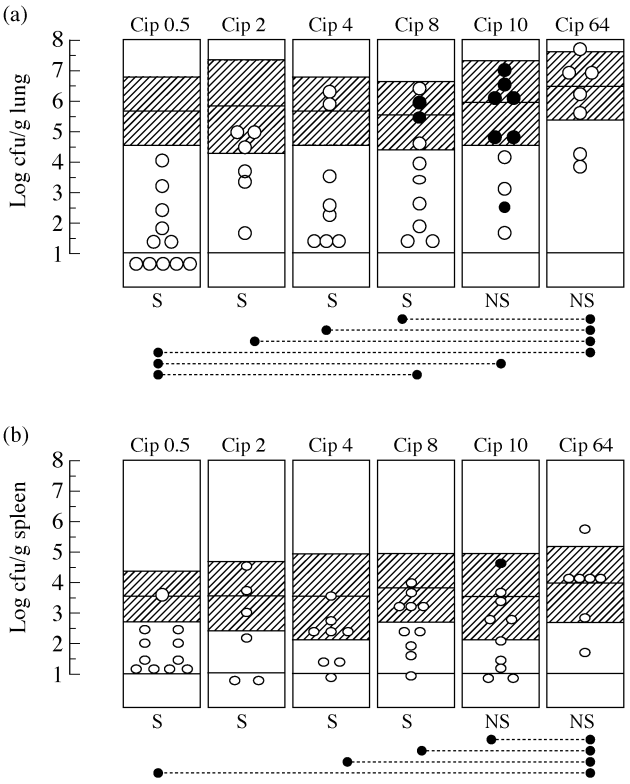

Figure 3. Outcome of experimental pneumonia caused by pneumococci with various susceptibility levels to fluoroquinolone and treated with human-like levofloxacin 500 mg iv twice a day for 48 h. The six strains are described in Table 1. The hatched boxes represent the pulmonary (a) and splenic (b) bacterial densities (mean ± S.D.) of untreated animals. Each circle represents the bacterial density of a single treated animal. The black circles represent the bacterial densities of mutants. NS (non-significant) and S (significant, $P < 0.05$) indicate significance between treated and untreated animals within each group analysed. The horizontal dotted lines indicate significant differences between groups.

It is critical to investigate situations for which clinical trials cannot provide adequate response simply because the antibiotic phenotype of pathogens is not known before antibiotic prescription and/or the probability of a pathogen with a reduced susceptibility profile is low. Such situations exist for the empirical treatment of pneumococcal pneumonia with levofloxacin. Indeed, fluoroquinolones with extended activity to Gram-positive pathogens are included in the therapeutic strategy for community-acquired pneumonia in many countries, and pneumococci resistant to ciprofloxacin or ofloxacin, but susceptible to levofloxacin, are increasingly reported.[10–16,44,45] Therefore, we used such strains that possessed the most frequent mutations and mechanisms of resistance observed in humans.[43,46]

In these conditions, our model of pneumococcal pneumonia in an immunocompetent animal with a human adapted treatment can potentially provide some useful observations. Indeed, this model can be considered as severe, since all untreated animals were septicaemic (as shown by a positive spleen culture) and always had more than two pulmonary lobes involved and pleural effusion to a level that would have some clinical impact in humans.[2,32,47–50] Keeping in mind that the strains used were resistant to penicillin, it is noteworthy that both the ciprofloxacin-resistant and -susceptible strains did induce experimental pneumonia with the same intensity (in terms of lesion and bacteriological content).

Our findings are in accordance with the conclusions of clinical trials in which levofloxacin treatment of pneumococcal pneumonia was associated with an 80% cure equivalent to the comparators.[7–9,51] In our study, the bacterial reduction induced by the treatment was very important for the fully susceptible strain Cip0.5. However, the pulmonary bacterial reduction was less for the less susceptible strains Cip2 (MIC 1 mg/L of levofloxacin) and Cip4 (MIC 1.75 mg/L, only with efflux of levofloxacin). This *in vivo* efficacy of levofloxacin could be anticipated by the bactericidal effect as observed by the *in vitro* killing curve data. It was notable that this antibacterial effect was associated with a reduction of the pulmonary hyper-permeability when animals were infected with the most susceptible strain.

The Cip8 strain exhibited the same MIC of levofloxacin as for Cip4 but the former strain possessed a *parC* mutation and no significant efflux of levofloxacin. The *in vivo* Cip8 bacterial reduction with levofloxacin was lower than for Cip4 pneumonia, which correlates with the delay in *in vitro* killing. Furthermore, *in vivo* mutations were observed only with Cip8 infection. These mutations were probably favoured by the presence of a *parC* mutation.[18,52]

The *in vivo* results observed with the strain Cip10, which possessed both an efflux mechanism for levofloxacin and a *parC* mutation, were concordant with the *in vitro* killing curve, which showed an incomplete killing and a regrowth due to mutants. This situation was very similar to that observed in the endocarditis model of infection with borderline-susceptible streptococci.[53,54] Furthermore, clinical failure of levofloxacin treatment of pneumococcal infection has been reported.[55–58]

The presence of a *parC* mutation was found to be a poor surrogate marker for *in vitro* fluoroquinolone resistance,[59] which our *in vitro* killing data confirmed. However, in our animal model, we found that the presence of a *parC* mutation was significantly associated with the absence of an *in vivo* killing effect; this could be explained by the appearance of mutants.

Surprisingly, we also found that the presence of significant efflux of ciprofloxacin was significantly associated with a reduced antibacterial effect of the simulated human-like treatment with levofloxacin. This was not anticipated from our results of *in vitro* killing. However, although the two strains Cip2 and Cip4 were susceptible to levofloxacin, a significant *in vitro* efflux of levofloxacin (31% and 16%, respectively)

Table 4. Pharmacodynamic parameters obtained in rabbits with experimental pneumonia induced with pneumococci of various susceptibility levels to fluoroquinolone for simulated human-like treatment using levofloxacin

Group of treated animals by strain	Pharmacodynamic indices		
	AUC/MIC[a]	C_{max}/MIC[a]	$\%T > MIC$[a]
Cip0.5	86 ± 30	11 ± 2	91 ± 5.5
Cip2	56 ± 22	9.4 ± 4	77 ± 9
Cip4	33 ± 15	4 ± 1.5	45 ± 10
Cip8	20 ± 9	2.1 ± 1	27 ± 9
Cip10	24 ± 5	2.5 ± 0.7	33 ± 5
Cip64	2.8 ± 1.6	0.4 ± 0.2	0.6 ± 0.4

[a]Significantly different between groups (ANOVA, $P < 0.05$).

with increased MICs was present, and therefore could explain the reduction of therapeutic efficacy.[23,60]

Since the three PK–PD indices were highly correlated, it was difficult to find only one good parameter that was predictive of the antibacterial effect. This could also be explained by the immunocompetent status of the animals. However, we found that AUC/MIC and C_{max}/MIC were significantly associated with the antibacterial effect, which is concordant with our current knowledge. Otherwise, it is noteworthy that $T > MIC$ was significantly associated with an *in vivo* bactericidal effect. This observation is not usual[61] and further specific investigations are warranted to elucidate the PD response of strains with reduced susceptibility to fluoroquinolones.

From the clustering analysis, an AUC/MIC ratio of at least 32 was identified and was associated with a 95% chance of a significant antibacterial effect. This value is close to those obtained *in vitro*[62] but higher than that obtained with the thigh infection model in mice.[63] More importantly, this value is very similar to 33.7, this being the value of AUC/MIC found to be associated with a microbiological response of 100% in patients with respiratory tract infections due to *S. pneumoniae* and treated with fluoroquinolones.[64]

In this *in vivo* study, all mutants were detected in animals infected with strains possessing a *parC* mutation. This observation is concordant with *in vitro* data.[26,52] Furthermore, these *in vivo* mutants appeared for AUC/MIC ratios between 13 and 31. Thus, from our results, the PD target for levofloxacin treatment is to obtain an AUC/MIC ratio of at least 30–35, which could be attained in 80% of levofloxacin-treated patients.[65] The incidence of two of the most prevalent *parC* mutations is increasing but remains <5% in levofloxacin-susceptible pneumococci in the USA.[66] So, the clinical risk of mutation would seem to be low. However, the prevalence of pneumococcal strains resistant to ciprofloxacin (and with reduced susceptibility to levofloxacin) is increasing in Europe.[45,67] Consequently, and considering that a standard treatment of levofloxacin is associated with a mean AUC_{0-24} of 50 mg·h/L, our results would predict therapeutic failure for patients infected with strains having levofloxacin MICs > 1.5 mg/L (corresponding to 4 mg/L of ciprofloxacin).

We conclude that our model of pneumococcal pneumonia with simulated human-like treatment has raised useful arguments in the discussion for the determination of *in vivo* MIC breakpoints,[68–71] since it allows a PK–PD analysis for each human-like treated animal with pulmonary infectious disease resembling human pneumococcal pneumonia.

In this study, we showed that human-like treatment with levofloxacin 500 mg twice a day for 48 h was quite efficacious for pneumococcal pneumonia caused by bacteria having MICs up to 1.5 mg/L of levofloxacin and 4 mg/L of ciprofloxacin. This treatment was associated with the appearance of mutants when strains had reduced susceptibility. Thus, we suggest that it may be worth reviewing the 2 mg/L susceptibility breakpoint of levofloxacin.

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