# Effect of Low-Level Resistance on Subsequent Enrichment of Fluoroquinolone-Resistant *Streptococcus pneumoniae* in Rabbits

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***Background.*** We measured the effect of low-level fluoroquinolone resistance in *Streptococcus pneumoniae* on the development of high-level resistance within the context of the mutant selection window.

***Methods.*** Rabbits infected with *S. pneumoniae* were treated with ciprofloxacin or moxifloxacin concentrations that simulated pharmacokinetics in treated humans; bacteria obtained from lungs were examined for fluoroquinolone susceptibility.

***Results.*** Ciprofloxacin enriched resistant mutants from a wild-type strain; moxifloxacin did not. However, moxifloxacin enriched resistant mutants from a *parC* mutant; the drug concentration at the top of the selection window was determined.

***Conclusions.*** A *parC* resistance mutation facilitates the enrichment of high-level resistance, as was predicted by in vitro measurements.

*Streptococcus pneumoniae* is the leading cause of community-acquired bacterial pneumonia, despite immunization and a variety of antimicrobial therapies. Over the past decade, susceptibility to $\beta$-lactams and macrolides has drastically declined [1]. As a consequence, many physicians have switched to fluoroquinolone treatment of pneumococcal pneumonia, and now fluoroquinolone resistance is being observed [2]. In some locales, ciprofloxacin-resistant *S. pneumoniae* has even reached the dissemination phase [3]. A new strategy for antibiotic use may be required to preserve the utility of existing agents and to assure a long life span for new compounds.

One approach for restricting resistance is to avoid conditions that enrich resistant bacterial subpopulations [4, 5]. Such conditions have been defined in vitro for fluoroquinolones with a variety of bacteria. In general, fluoroquinolone-resistant mutants are enriched when drug concentrations fall within a range called the mutant selection window [4]. The lower boundary of the window is approximated by the $MIC_{99}$, the minimal concentration that blocks growth of 99% of the cells in a culture. The upper boundary is the MIC of the least-susceptible single-step mutant, a value called the mutant prevention concentration (MPC), because, above this concentration, 2 concurrent resistance mutations must be acquired for growth. Conditions that expand the selection window, such as the acquisition of a low-level *parC* (DNA topoisomerase IV) resistance allele by *S. pneumoniae,* are expected to facilitate the enrichment and amplification of high-level resistant mutants, because bacterial exposure to the antimicrobial agent will be inside the window longer [6]. Moreover, quinolone lethality is expected to be lower [6], and the frequency at which gyrase mutants are enriched will be higher [6, 7]. The present work shows that predictions based on in vitro observations apply in an animal model, which supports the approach of evaluating chemotherapies for antimutant activity and emphasizes the importance of halting the development of low-level resistance.

***Materials and methods.*** *S. pneumoniae* (serotype 9V) strains included a fluoroquinolone-susceptible, penicillin-resistant clinical isolate (16089) and 2 laboratory derivatives selected by exposure to ciprofloxacin—strain MS1A, a reserpine-sensitive efflux strain, and MR3B4, a *parC* Ser-79 to Tyr variant [8]. *S. pneumoniae* was grown at 37°C in 5% $CO_2$ in brain-heart infusion (BHI) broth (BioMérieux), on either sheep-blood agar plates (BioMérieux) or tryptic soy agar that contained 5% sheep blood (TSAB; BioMérieux). Ciprofloxacin and moxifloxacin were products of Bayer AG.

The MIC was determined by agar dilution by use of an inoculum of $10^5$–$10^6$ cfu/mL (this is ~2-fold higher than the $MIC_{99}$) [6]. The MPC was determined by applying 0.2 mL of bacterial culture that contained $\geqslant 10^{10}$ cfu/mL to TSAB plates that contained fluoroquinolone at various concentrations. Plates were examined after incubation for 48 and 72 h. The MPC was defined as the lowest fluoroquinolone concentration that prevented bacterial colony formation at 48 h. Identical results were obtained from experiments performed in triplicate.

For animal infection, *S. pneumoniae* was grown in BHI broth

Received 6 February 2004; accepted 10 April 2004; electronically published 8 September 2004.

Presented in part: 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, 14–17 September 2003 (abstract A-1321).

Financial support: The Medex Society, Fondation pour la Recherche Médicale; National Institutes of Health (grant AI 35257); Bayer AG; Bristol-Myers-Squibb.

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**The Journal of Infectious Diseases 2004;190:1472–5**
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0022-1899/2004/19008-0014$15.00

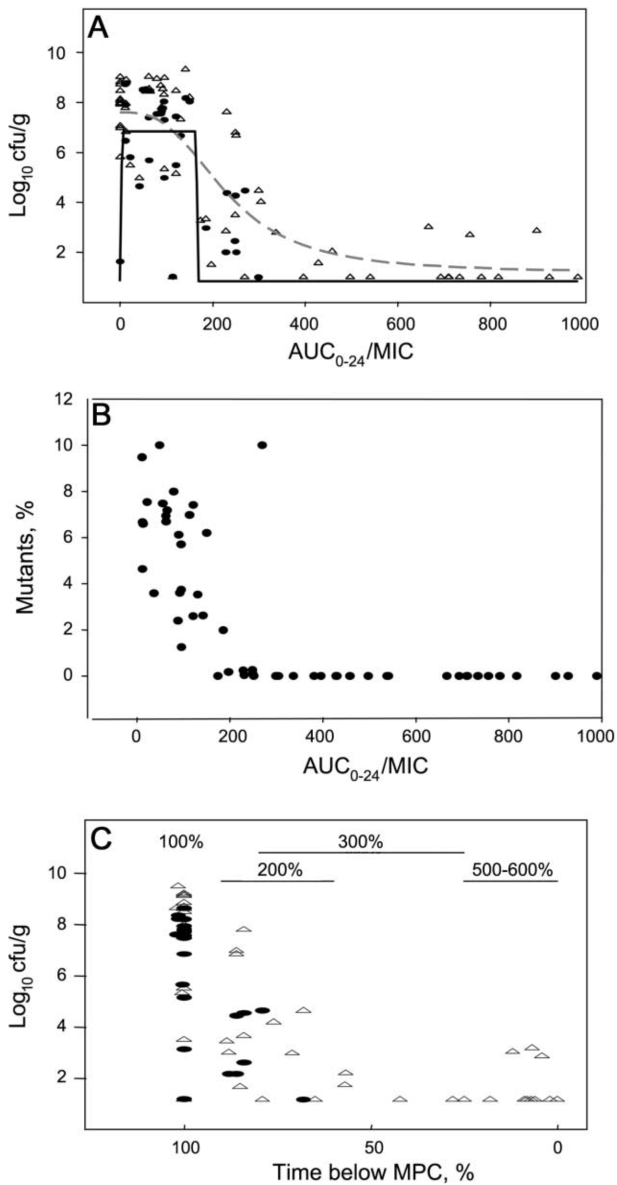


**Figure 1.** Effect of moxifloxacin treatment on *Streptococcus pneumoniae* recovered from rabbit lung. Immunocompetent rabbits were infected with a *parC* mutant (strain MR3B4) and then treated with various doses of moxifloxacin. Bacteria were obtained from lungs, and the nos. of bacteria per gram of lung tissue were determined as either resistant (*black circles,* $MIC_{48}/MIC_0 > 4$) or parental (*white triangles,* $MIC_{48}/MIC_0 = 1$) cells. Some animals had both resistant and parental cells. *A,* Relationship between bacterial recovery and effective moxifloxacin concentration, expressed as area under the drug concentration–time curve $(AUC)_{0-24}$/MIC. Dashed line, bacteria showing no increase in MIC (parental bacteria; $E_{max}$ regression curve, $R^2 = 0.63$). Solid line, resistant bacteria (peak regression curve, $R^2 = 0.83$). *B,* Relationship between fraction of cells recovered as mutant and moxifloxacin exposure. Resistant bacteria recovered in panel *A* are expressed as a fraction of the total recovered from each rabbit. *C,* Relationship between bacterial recovery and the fraction of time during which the free serum concentration was below the mutant prevention concentration (MPC) determined in vitro. *Black circles,* resistant mutants; *white triangles,* wild-type cells; *numbered bars,* moxifloxacin dose, given as a percentage of the standard human dose.

and transferred to agar plates to obtain colonies, of which 25–30 were combined and grown in 9 mL of BHI for 6 h. These cultures were used to form bacterial lawns by incubation on TSAB for 18 h. Lawns were resuspended in physiological saline at a final concentration of $10^{10}$ cfu/mL.

Two silicon catheters (Sigma Medical) were inserted into the jugular vein of immunocompetent rabbits, as described elsewhere [8]. Bacterial pneumonia was induced 24 h after jugular catheterization by endobronchial challenge under laryngoscopic control with 0.5 mL of saline that contained $10^{10}$ cfu/mL *S. pneumoniae* (the detection of rare mutants requires large inocula). Fluoroquinolone treatment began 4–5 h later through the first central venous catheter at infusion rates controlled by programmable computer software to simulate drug pharmacokinetics in human serum with either (1) the simulated standard 400-mg dose of ciprofloxacin given intravenously twice daily for 1 day ($C_{max} = 4$ mg/L; area under the 24-h drug concentration–time curve [$AUC_{0-24}$], 11.4 mgh/L) [9] or (2) various doses of moxifloxacin expressed as a percentage of the standard 400-mg intravenous dose once daily for 2 days ($C_{max} = 4.5$ mg/L; $AUC_{0-24}$, 35–40 mgh/L) [10].

Rabbits were anesthetized and killed 4–5 h after the last infusion of fluoroquinolone. Each pulmonary lobe was weighed and homogenized in sterile physiological saline. Bacterial numbers were determined by plating 10-fold dilutions of organ homogenates on TSAB, followed by incubation for 24 h. For statistical comparisons of bacterial numbers in lungs, culture-negative lobes were assumed to contain 10 cfu/g tissue, because this was the limit of detection. When bacteria from the pulmonary homogenates grew on TSAB plates that contained fluoroquinolone at 2 and 4 times the initial MIC, additional MIC determinations were made by use of 0.25 mg/L increments in the fluoroquinolone concentration. Nucleotide sequence determination of the quinolone resistance–determining regions (QRDRs) of *parC, parE, gyrA,* and *gyrB* [11] was done by use of automated sequencing (Genome Express).

For each animal, the serum concentration of fluoroquinolone was determined by bioassay from blood samples obtained through the second central catheter. A disc agar-plate bioassay method was used with antibiotic medium II (Difco Laboratories) and *Escherichia coli* NIJJHC2 as the indicator organism. The assay was linear between 0.5 and 7 mg/L ($R^2 = 0.99$); within-day and between-day coefficients of variation for replicates were <5%. The binding of fluoroquinolone to plasma protein was 30%, as determined by a membrane filtration method [10]. Results were expressed as the free fraction.

For moxifloxacin, doses varied such that the $C_{max}$ was $0.47 \pm 0.01$ to $22.6 \pm 4.3$ mg/L and the $AUC_{0-24}$ was $2.9 \pm 0.1$ to $215 \pm 24$ mgh/L. As with humans [10], no variation in drug half-life was associated with the antibiotic dose; thus, a linear

**Table 1. Effect of moxifloxacin on loss of fluoroquinolone susceptibility by a *parC* resistance mutant of *Streptococcus pneumoniae* strain MR3B4.**

| | | | Bacteria recovered from lungs | | |
|---|---|---|---|---|---|
| | | | | Bacterial concentration | |
| Simulated dose of moxifloxacin,[a] % | Free $AUC_{0-24}/MIC_0$, h | Time below MPC,[b] % | Proportion (%)[b] of animals with resistant bacteria | Total, $log_{10}$ cfu/g | Resistant bacteria,[c] % |
| 0 | 0 | 0 | 0/13 (0) | 8 ± 0.8 | 0 |
| 6 | 12 ± 0.8 | 100 | 4/4 (100) | 8 ± 0.9 | 69 |
| 25 | 49 ± 18 | 100 | 5/5 (100) | 7.7 ± 1.7 | 62 |
| 50 | 83 ± 16 | 100 | 5/5 (100) | 8 ± 1.7 | 17 |
| 100 | 120 ± 30 | 100 ± 0.8 | 11/11 (100) | 7.1 ± 2.6 | 45 |
| 200 | 260 ± 41 | 81 ± 7.1 | 6/8 (75) | 4.5 ± 2.1 | 12 |
| 300 | 470 ± 120 | 48 ± 23 | 0/7 (0) | 1.7 ± 1.1 | 0 |
| 500 | 600 ± 240 | 23 ± 37 | 0/4 (0) | 2.2 ± 1.1 | 0 |
| 600 | 860 ± 97 | 11 ± 9.6 | 0/6 (0) | 1.3 ± 0.7 | 0 |

**NOTE.** Data are mean ± SD unless otherwise indicated. AUC, area under the 24-h drug concentration–time curve.

[a] Expressed as a percentage of the standard dose equivalent in humans to 400 mg given intravenously once daily.

[b] Percentage of the treatment time during which the free serum concentrations fell below the mutant prevention concentration, measured in vitro with agar plates.

[c] Resistant bacteria were defined as $MIC_{48}/MIC_0 \geq 4$.

relationship ($R^2 = 0.98$) was observed between the simulated dose and the $AUC_{0-24}$ for free (unbound) drug.

***Results.*** To establish an in vivo model for the enrichment of bacterial mutants, 4 rabbits were each infected with $5 \times 10^9$ cfu of wild-type *(wt) S. pneumoniae,* strain 16089 (ciprofloxacin MIC = 0.5 mg/L; MPC = 4 mg/L). Rabbits were then given 2 intravenous treatments of ciprofloxacin that simulated human dosing and pharmacokinetics. Approximately $5 \times 10^8$ cfu *S. pneumoniae*/g lung tissue were recovered, of which 1% exhibited an increased MIC (4 mg/L). DNA from 2 of these organisms had nucleotide sequence changes in the QRDR of *parC* that was expected to change Ser-79 to Phe. A comparable number of bacteria was recovered from 13 control rabbits that had not been given drugs. None of the control animals had bacteria with elevated MICs. Thus, resistant mutants were readily recovered when a *wt* pneumococcal infection was treated with ciprofloxacin such that serum drug pharmokinetics were equivalent to those attained in human therapy.

Next, 42 rabbits were infected with either *wt* (16089) or efflux mutant (MS1A) bacteria and then treated with moxifloxacin at doses of 15%–100% of the standard human treatment, once daily for 2 days (MIC = MPC = 0.125 mg/L with *wt* and 0.25 mg/L with the efflux mutant). Doses >25% of the standard human treatment reduced bacterial numbers by almost 7 and 6 orders of magnitude with the *wt* and the efflux strains, respectively (data not shown). Regardless of dose or strain tested, surviving bacteria exhibited no increase in MICs. Thus, 2 doses of moxifloxacin are considerably more effective than are 2 doses of ciprofloxacin at restricting the recovery of *S. pneumoniae.*

Because in vitro results had predicted that a low-level *parC* resistance mutation would facilitate the enrichment of resistant gyrase mutants by moxifloxacin [6], we infected rabbits with the *parC* mutant strain MR3B4 (moxifloxacin MIC = 0.25 mg/L; MPC = 4 mg/L) and measured mutant enrichment by various doses of moxifloxacin (6%–600% of the standard human dose). Low doses failed to reduce the bacterial load but did enrich resistant mutants (figure 1*A*), as was seen with ciprofloxacin treatment of *wt* cells. Doses that were at least twice the simulated human standard were required to reduce the total number of bacteria and fluoroquinolone-resistant mutants (MIC≥4 mg/L) recovered from rabbit lungs (figure 1*A* and 1*B*, table 1). DNA from 2 of the resistant organisms exhibited alterations in *gyrA* that are expected to change Ser-81 to Phe in the GyrA protein.

A sharp drop in the recovery of resistant mutants occurred at $AUC_{0-24}$s and MICs of 200–300 (figure 1*A* and 1*B*, averages in table 1). Using classification and regression tree analysis (Answer Tree software; SPSS), the drug exposure ($AUC_{0-24}$/MIC) that limited the recovery of resistant mutants was 300 h ($P = .04$), a value corresponding to 220% the standard moxifloxacin dose for humans. The recovery of mutants at high moxifloxacin doses dropped sharply as the time below the MPC decreased (figure 1*C*, averages in table 1).

***Discussion.*** The present work establishes that a *parC,* but not a ciprofloxacin-resistant, efflux mutation in *S. pneumoniae* facilitates the enrichment of resistant mutants in rabbits treated with moxifloxacin. The results of earlier in vitro studies had shown that the acquisition of a *parC* fluoroquinolone-resistance mutation by *S. pneumoniae* decreases susceptibility ~2-fold for moxifloxacin, increases by 10-fold the selection window for obtaining mutants, increases the frequency at which *gyrA* mutants are recovered by 3 orders of magnitude, and decreases

the lethal activity by 3-fold [6, 7]. These in vivo and in vitro results, together with a surveillance study that reported a prevalence of 4% for *parC* mutants [12], suggest a sharp increase in high-level fluoroquinolone resistance and treatment failure [13, 14] if serious efforts are not made to halt the enrichment of additional *parC* mutants.

The present work also supports the mutant selection-window hypothesis, an idea that provides a rationale for restricting mutant growth [4]—the number of mutants recovered dropped sharply as the time the serum drug concentration exceeded the MPC (figure 1*C*), the upper boundary of the selection window. This drug concentration threshold occurred at $AUC_{0-24}/MIC = 300$. We did not define the lower boundary of the selection window, because resistant mutants were enriched even at the lowest dose, which placed moxifloxacin concentrations above the MIC for 13% of the time.

The ease at which *parC* resistance mutants were recovered from rabbits after the treatment of *wt* infection with ciprofloxacin supports the early decision to discourage the use of ciprofloxacin for pneumococcal disease [13]. Levofloxacin, another compound that preferentially enriches *parC* mutants, is more active, and *parC* mutants are considered to be susceptible to this drug. However, levofloxacin-resistant *parC gyrA* double mutants have been readily obtained in vitro [6], in the rabbit model of pneumonia [8], and in humans [14] when the infecting strain contains a *parC* mutation. A key to successful fluoroquinolone use may involve restricting the enrichment of first-step mutants. That may be easier with moxifloxacin than with levofloxacin, because a loss of susceptibility to the former appears first through *gyrA* mutations, which are recovered less often with moxifloxacin than are *parC* mutants with levofloxacin [6, 7].

In summary, when rabbit infection caused by a *parC* mutant of *S. pneumoniae* was treated with moxifloxacin at the standard human dose, the drug concentration was below the MPC for 100% of the time, and mutants considered to be resistant were recovered from all of the treated animals. To prevent the same phenomenon from happening in humans when bacterial burdens are high or, perhaps, when patient numbers are very large, efforts must be made to (1) minimize the use of quinolones that readily enrich *parC* mutants, (2) routinely test *S. pneumoniae* isolates for *parC* mutations, and (3) avoid fluoroquinolones when the probability of a *parC* mutant infection is high, even though such strains may be considered susceptible according to break-point criteria.


## Acknowledgments

We thank Joseph Blondeau, Marila Gennaro, Samuel Kayman, Xinying Li, Tao Lu, Lionel Piroth, and Xilin Zhao, for critical comments on the manuscript.


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