The Mutant Selection Window in Rabbits Infected with *Staphylococcus aureus*

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**Background.** The mutant selection window hypothesis, originally based on agar plate assays, may lead to new antimicrobial dosing strategies that severely restrict the acquisition of resistance. However, it has not been directly tested in an animal model of infection.

**Methods.** Local infection with *Staphylococcus aureus* was established in rabbits, and the infected animals were treated orally with various doses of levofloxacin. Changes in levofloxacin concentration, levofloxacin susceptibility, and counts of total and resistant viable bacteria were monitored at the site of infection.

**Results.** *S. aureus* lost levofloxacin susceptibility when drug concentrations at the site of infection fluctuated between the lower and upper boundaries of the window, defined in vitro as the minimum inhibitory concentration (MIC) 99 and the mutant prevention concentration (MPC), respectively. The upper boundary of the selection window in vivo was estimated as an AUC 24/MPC value of ∼25 h, where AUC 24 is the area under the drug concentration time curve in a 24-h interval. The lower boundary was estimated as an AUC 24/MIC value of ∼20 h.

**Conclusions.** The mutant selection window exists in vivo, and its boundaries fit well with those determined in vitro. Maintenance of antimicrobial concentrations above the window is expected to suppress the outgrowth of resistant mutant subpopulations.

Antimicrobial resistance arises from a variety of factors, including drug overuse [1, 2] and drug misuse [3], both in the environment and during therapy [4]. Even the commonly accepted treatment strategy of killing susceptible pathogens [5] contributes to the problem by allowing selective amplification of resistant mutants that are present as small subpopulations before treatment [6]. Consequently, the acquisition of resistance can occur concurrently with the eradication of susceptible cells [7, 8]. The drug-concentration range in which mutants selectively amplify (the mutant selection window) has been defined quantitatively by use of agar plate assays [9–11]. The lower boundary of the selection window is the minimum concentration that blocks susceptible cell growth (approximated by MIC 99); the upper boundary is the minimum concentration that inhibits growth of the least-susceptible single-step mutant subpopulation, a value called the “mutant prevention concentration” (MPC) [12]. Before the mutant selection window can be used as a framework for the design of antimicrobial therapy, the existence of the window must be demonstrated in vivo. Moreover, agar plate assay–derived window boundaries must be correlated with the window dimensions directly observed in vivo so that clinical laboratory data can be applied to individual patients. The upper boundary of the window has been approximated in a rabbit pneumococcal pneumonia model in which plasma pharmacodynamic parameters were related to the recovery of resistant mutants in the lungs [13–15]. However, a lower boundary has not been demonstrated, nor has either boundary been defined using drug concentrations at the site of infection.

Localized infections are particularly well suited for testing the mutant selection window hypothesis, because both drug concentration and the selective am-
plification of resistant mutants can be directly measured at the site of infection. One such system is the tissue-cage infection model [16–18], in which a foreign-body device (e.g., a hollow plastic ball with holes in its surface [a Wiffle ball]) is surgically implanted under the skin of an animal. In rabbits, the surface of the ball becomes encapsulated by connective tissue 2–4 weeks after implantation, and the interior fills with fluid (tissue-cage fluid). Bacterial cultures injected into the ball remain there until eliminated by host defenses and antimicrobial treatment. Rabbits having tissue-cage fluid was withdrawn from each plastic ball for a preimplanted plastic ball. Two days after infection, 0.5 mL of concentrated in 1 mL of 0.9% NaCl and injected into each rabbit through a dorsal midline incision under aseptic conditions. After surgery, the rabbits were treated with intramuscular penicillin (100,000 IU/kg) twice daily for 3 days to prevent infection. By 4 weeks after implantation, each plastic ball had become sealed with a thin layer of connective tissue and had filled with clear, yellowish tissue-cage fluid. Approximately 1.5 × 10^6 cfu of exponentially growing S. aureus culture was concentrated in 1 mL of 0.9% NaCl and injected into each preimplanted plastic ball. Two days after infection, 0.5 mL of tissue-cage fluid was withdrawn from each plastic ball for a viable-bacteria count. Rabbits having >1 × 10^9/mL viable bacterial cells were treated with various doses of levofloxacin. The experimental protocol was approved by the Research Animal Care and Use Committee of the PLA General Hospital.

**Pharmacokinetic measurements.** Rabbits were administered levofloxacin at 0, 5, 10, 20, 25, 30, or 40 mg/kg of body weight orally. Resistant mutants were selected predominantly when drug concentrations were maintained inside a concentration window having boundaries that fit well with those determined using agar plate assays. These data provide a clear demonstration of the mutant selection window in vivo and support arguments for how antimicrobial treatment regimens can be adjusted to severely restrict the amplification and enrichment of resistant mutants.

**Figure 1.** Effect of levofloxacin dose on bacterial survival in the tissue-cage model. Tissue-cage (perforated plastic ball) implantation and *Staphylococcus aureus* infection were done as described in Materials and Methods. Three days after infection (indicated by the arrow), various oral doses (0, 5, 10, or 30 mg/kg of body weight; the no. of rabbits in each study group is indicated in the key) of levofloxacin were administered once daily for 5 days. Bacterial colony-forming units in tissue-cage fluid was monitored at 24-h intervals beginning 1 day before the initiation of levofloxacin treatment and ending 2 days after the termination of levofloxacin treatment.

**MATERIALS AND METHODS**

**Antimicrobials, chemicals, and enzymes.** Penicillin, as a sodium salt for injection, was purchased from North China Pharmaceutical Group Corporation. Levofloxacin for injection was from Lizhu Pharmaceutical Corporation, and levofloxacin standard and reserpine were from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing). Acetonitrile (chromatography grade) was from Fisher Scientific. RNase A and Triton X-100 were from Amresco, and lysozyme was from BBI-Biotech Research Laboratories. Taq DNA polymerase was from Promega, dNTP was from Takala, and oligonucleotide primers were from AuGCT. Other chemicals, which were analytical grade, were purchased from Beijing Chemical Corporation.

**Bacterial growth and drug susceptibility.** *S. aureus* strain ATCC 25923 was grown in Muller-Hinton broth or on Muller-Hinton agar. MIC, MIC99, and MPC were determined as described elsewhere [19, 20]. Briefly, bacterial cultures were grown overnight at 37°C, serially diluted, and applied to agar plates containing various concentrations of levofloxacin. After incubation at 37°C for 24–72 h, bacterial colonies were counted, and the fraction relative to the bacterial inoculum was calculated. Drug concentrations that inhibited growth by 99% and 99.99% were defined as MIC99 and MIC, respectively. The MPC was the drug concentration that prevented colony recovery from 1 × 10^10 cells applied to multiple levofloxacin-containing agar plates.

**Tissue-cage infection model.** Female New Zealand White rabbits weighing 2.5–3 kg each were supplied by the PLA General Hospital Experimental Animal Center. The rabbits, which were housed individually, were allowed free access to food and water. Before the implantation of the perforated plastic balls, rabbits were anesthetized by use of ketamine (40 mg/kg) and xylazine (5 mg/kg) intramuscularly. Then a plastic ball (43 mm in diameter, with a volume of 34 mL) was implanted into each rabbit through a dorsal midline incision under aseptic conditions. After surgery, the rabbits were treated with intramuscular penicillin (100,000 IU/kg) twice daily for 3 days to prevent infection. By 4 weeks after implantation, each plastic ball had become sealed with a thin layer of connective tissue and had filled with clear, yellowish tissue-cage fluid. Approximately 1.5 × 10^6 cfu of exponentially growing *S. aureus* culture was concentrated in 1 mL of 0.9% NaCl and injected into each preimplanted plastic ball. Two days after infection, 0.5 mL of tissue-cage fluid was withdrawn from each plastic ball for a viable-bacteria count. Rabbits having >1 × 10^9/mL viable bacterial cells were treated with various doses of levofloxacin. The experimental protocol was approved by the Research Animal Care and Use Committee of the PLA General Hospital.

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Figure 2. Effect of levofloxacin concentration on loss of susceptibility and mutant enrichment. Tissue-cage (perforated plastic ball) implantation and Staphylococcus aureus infection were done as described in Materials and Methods. Rabbits having $>1 \times 10^4$ cfu/mL S. aureus in tissue-cage fluid 2 days after infection were treated with various oral doses of levofloxacin once daily for 5 days beginning 3 days after infection. The dosage protocol for each panel was as follows: A1, all received 5 mg/kg; A2, 2 received 5 mg/kg and 1 received 10 mg/kg; A3, 4 received 5 mg/kg and 3 received 10 mg/kg; A4, 2 received 20 mg/kg and 3 received 25 mg/kg; A5, 1 each received 20, 30, and 40 mg/kg; and A6, 1 received 30 mg/kg and 3 received 40 mg/kg. Levofloxacin concentration in tissue-cage fluid was monitored at the indicated times after the administration of each dose (panels A1–A6). The boundaries of the mutant selection window (i.e., the MIC$_{99}$ and the mutant prevention concentration [MPC]) were determined with the S. aureus inoculum by agar plate assays. The double-headed arrow indicates the mutant selection window. Tissue-cage fluid was sampled for bacteria at 24-h intervals for 6 days starting immediately before the administration of the first dose of levofloxacin. Loss of susceptibility (panels B1–B6) was monitored as an increase in MIC averaged for all rabbits in the group. The no. of rabbits that had lost levofloxacin susceptibility by the indicated time point is indicated under the curves in panels B2–B4; error bars tended to be large because some rabbits showed no MIC change and others exhibited substantial change. Asterisks indicate data points that are not significantly different from baseline values. The fraction of resistant mutants (panels C1–C6) in each group of rabbits was determined daily as the no. of colonies grown on levofloxacin-containing agar (0.125 mg/L [1 MIC]) relative to the no. that grew on drug-free agar. The MIC$_{99}$ (0.1 mg/L) and the MPC (0.8 mg/L) are shown as solid and dashed lines, respectively (panels A1–A6). Nos. at the top of the figure indicate the no. of rabbits in each study group. The dashed line in panel C6 indicates that the no. of resistant mutants decreased to below the limit of detection (10 cfu/mL) in 3 of the 4 rabbits after the first dose; the remainder of the data points are for a single rabbit.
levofloxacin and ciprofloxacin was then determined in the presence or absence of reserpine at 20 mg/L to assess the contribution of efflux. DNA extracted from these mutants was subjected to amplification by polymerase chain reaction, and the nucleotide sequence of the quinolone-resistance-determining regions of \textit{parC} (gyrA) and \textit{gyrA} were determined as described elsewhere [19, 22].

\section*{Statistical analysis.}
Fisher's exact test was used for statistical analysis of the PK/PD data, with an infected but untreated set of rabbits as a control. \(P < .05\) was considered to be statistically significant.

\section*{RESULTS}

\subsection*{Tissue-cage infection model.}
Rabbits each received a single implant with a perforated plastic ball. When \(\sim 1.5 \times 10^9\) bacteria were injected into an implanted ball, no severe illness or distress occurred during a 10-day observation period. Bacterial concentrations remained constant at \(\sim 1 \times 10^7\) cfu/mL when rabbits were treated orally with saline once daily for 5 days (figure 1, \textit{white squares}). In a preliminary experiment, oral levofloxacin was administered daily for 5 days, beginning at day 3 after infection (figure 1, \textit{arrow}). Treatment was followed by a 2-day untreated period to allow outgrowth of residual bacteria. Doses of levofloxacin at 5 or 10 mg/kg reduced bacterial numbers for the first 3–4 doses, but bacterial growth was observed late during treatment and during posttreatment observation (figure 1, \textit{circles}). Levofloxacin at 30 mg/kg caused bacterial numbers to decrease throughout treatment and to remain low during the growth recovery phase (figure 1, \textit{black squares}). Thus, the bacterial response depended on the levofloxacin dose. Levofloxacin concentration, determined in samples of tissue-cage fluid collected at various time points over several days, is shown in figure 2 (panels A1–A6) relative to the boundaries of the mutant selection window (MPC [0.8 mg/L] and MIC\textsubscript{99} [0.1 mg/L]) measured by agar plate assays. These data showed that the rabbits could be grouped with respect to tissue drug concentrations (variation among rabbits precluded grouping according to the levofloxacin dose).

\subsection*{Effect of levofloxacin concentration on loss of susceptibility and mutant enrichment.}
Samples of \textit{S. aureus} in tissue-cage fluid were examined for susceptibility to levofloxacin after treatment with various doses. Increases in MIC\textsubscript{99} were readily observed (figure 2, panels B3 and B4) when levofloxacin concentrations were inside the selection window (figure 2, panels A3 and A4).

When levofloxacin concentrations were maintained either below the MIC\textsubscript{99} (figure 2, panel A1) or above the MPC (figure 2, panel A6), no MIC increase, either during or after therapy, was detected (figure 2, panels B1 and B6). When levofloxacin concentrations overlapped the lower window boundary (MIC\textsubscript{99}) such that they were above the boundary for \(<30\%\) of the treatment

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Effect of levofloxacin exposure on recovery of total and resistant bacteria. Concentrations of total bacteria and resistant mutants were determined in aliquots of tissue-cage fluid obtained at the indicated time points after the initiation of treatment. Representative examples are shown for rabbits in which the levofloxacin concentration was inside the mutant selection window, as determined in figure 2, panels A3 and A4. Circles and squares indicate different representative rabbits; black symbols represent the mutant concentration, and white symbols represent the total bacterial concentration. The dashed line indicates the limit of detection (10 cfu/mL). The pattern shown by the circles was observed in 5 rabbits; that by the squares was observed in 4 rabbits.}
\end{figure}
time (figure 2, panel A2), decreased susceptibility (MIC increase) was detected in only 1 of 3 rabbits (figure 2, panel B2). For drug concentrations that were above the MIC₉⁹ for >30% of the time but did not cross the upper boundary (MPC), susceptibility decreased in 9 of 9 rabbits, as indicated by MIC increases during levofloxacin treatment (all 7 rabbits depicted in panel A3 and 2 rabbits depicted in panel A4). When levofloxacin concentrations crossed the upper boundary of the window (MPC), loss of susceptibility did not occur if levofloxacin concentrations were above the MPC for >20% of the treatment time (8/8 rabbits; 7 depicted in panels A5 and A6, and 1 depicted in panel A4).

Dramatic increases (>1000 fold) in the fraction of cells that were mutant occurred when drug concentrations fell inside the selection window (figure 2, panels C3 and C4). Increases in the mutant fraction were more obvious than MIC changes in rabbits having levofloxacin concentrations fluctuating near the lower boundary (MIC₉⁹). The mutant fraction was expected to be more sensitive than the MIC to mutant subpopulation changes (figure 2, compare panel B2 with panel C2), because much larger populations of cells were tested (>1 × 10⁸ vs. 1 × 10⁶) with the former than with the latter, allowing mutant fractions to be monitored at much early stages (spontaneous background levels). Collectively, these data show that the selection window boundaries determined by agar plate assays fit well with in vivo determinations.

The fraction of mutants and the MIC can increase either by mutant amplification (outgrowth of mutant cells) or by mutant enrichment (killing of susceptible cells). To distinguish these situations, we determined the absolute number of both total and resistant bacteria. When drug concentrations were inside the selection window, the mutant response fell into 1 of 2 patterns, as the total population size first decreased and then gradually increased (figure 3, white symbols). In one pattern, the mutant fraction amplified throughout the experiment (figure 3, black circles). In the other, mutant numbers were initially either constant or decreased (figure 3, black squares), indicating that a fractional increase (figure 2, panels C2–C4) would be due to preferential loss of susceptible cells. After several days, mutant amplification was observed (figure 3, black squares). Thus, the selection of levofloxacin-resistant mutants in vivo probably arises from both mutant amplification and preferential loss of susceptible cells (mutant enrichment).

Selection of resistant mutants occurred 24–48 h earlier, when levofloxacin concentrations were in the lower portion of the selection window than in the upper portion, as indicated by both MIC and mutant-fraction increases (figure 2, compare panels B3 and C3 with panels B4 and C4). This result was expected because the fraction of mutants is higher near the bottom of the window in agar plate experiments [23]. Mutants selected in the lower and middle portions of the selection window tended to be nontopoisomerase mutants that exhibited increased efflux, because adding reserpine, an efflux inhibitor,
decreased the MIC for levofloxacin and ciprofloxacin (table 1). Mutants obtained in the upper portion of the selection window were less sensitive to reserpine, and 4 of 5 contained the parC Ser80Phe substitution that is commonly associated with quinolone resistance (table 1).

**Correlation of PK/PD indices with mutant enrichment and amplification.** PK/PD indices, such as AUC<sub>24</sub>/MIC (where AUC<sub>24</sub> is the area under the drug concentration time curve in a 24-h interval) and time above the MIC, provide an empirical way to relate antimicrobial dose and fluctuating drug concentrations to favorable treatment outcomes for bactericidal agents [24, 25]. These indices are thought to reflect the dynamic bacterial drug exposure when pathogens are exposed to fluctuating drug concentrations. Relationships between PK/PD indices, determined as steady-state values after the fifth dose, and loss of susceptibility are shown in table 2. For fluoroquinolones, AUC<sub>24</sub>/MIC is the index most commonly associated with restricting susceptible cell growth [25]. In only 1 of 6 rabbits was loss of susceptibility seen when AUC<sub>24</sub>/MIC was <20 h (table 2 and figure 2, panels A1 and A2). These data provide the first in vivo dynamic estimate of the lower boundary of the selection window. Loss of bacterial susceptibility occurred in 11 of 12 rabbits when AUC<sub>24</sub>/MIC was between 20 and 150 h (figure 2, panels A3 and A4, and table 2). No mutant enrichment was observed when AUC<sub>24</sub>/MIC exceeded 150 h (7 rabbits tested; figure 2, panels A5 and A6, and table 2).

AUC<sub>24</sub>/MPC, which is probably the appropriate index for the upper boundary of the selection window (see Discussion), restricted mutant selection when >25 h (7/7 rabbits; figure 2, panels A5 and A6, and table 2). Mutant selection was promoted when AUC<sub>24</sub>/MPC fell between 3 and 25 h (11/12 rabbits; table 2 and figure 2, panels A3 and A4).

Other pharmacodynamic indices also showed statistically significant correlations with the selection of resistance (table 2). When maximum concentration (C<sub>max</sub>)/MIC and C<sub>max</sub>/MPC were considered, the selection window extended from 1 to 8 and from 0.2 to 1.2, respectively. In another example, mutants were recovered from 12 of 17 rabbits when the levofloxacin concentration was above the MPC for <20% of the dosing interval. Correction for protein binding (mean ± SD, 29% ± 5%) had only a small effect on PK/PD indices and had little influence on the fraction of rabbits that acquired resistant bacteria in a particular PK/PD category.

**DISCUSSION**

As antimicrobial resistance becomes an increasingly serious problem, part of the clinical response will be to increase doses on an ad-hoc basis [26, 27]. The mutant selection window hypothesis provides a quantitative framework for raising doses. In a general sense, the selection window approach seeks to keep drug concentrations outside the selection window, whereas traditional strategies tend to place concentrations inside it. Concentrations inside the window are expected to allow the acquisition of resistance concurrently with eradication of susceptible pathogen populations, as we recently observed in patients with tuberculosis who were colonized by *S. aureus* and treated with rifampin [7]. A fundamental difference between the traditional MIC-based strategies and the MPC (selection window)–based approach is that the former requires bacteria to acquire only 1 mutation for growth in the presence of drug, whereas the latter requires 2 or more [9]. The MPC-based approach addresses the problem of resistant subpopulations existing before treatment, whereas traditional strategies do not.

To be clinically useful, the boundaries of the selection window need to be predictable on the basis of data obtained by clinical microbiological laboratories. Previous work has shown that static agar plate values of MIC and MPC fit well with

**Table 2. Correlation of pharmacokinetic/pharmacodynamic (PK/PD) parameters with selection of resistance.**

<table>
<thead>
<tr>
<th>PK/PD index, value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fraction of rabbits with resistant bacteria</th>
<th>P&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>AUC&lt;sub&gt;24&lt;/sub&gt;/MIC</td>
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<tr>
<td>&gt;150 h</td>
<td>0/7</td>
<td>NA</td>
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<tr>
<td>20–150 h</td>
<td>11/12</td>
<td>.009</td>
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<tr>
<td>&lt;20 h</td>
<td>1/6</td>
<td>.667</td>
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<td>AUC&lt;sub&gt;24&lt;/sub&gt;/MPC</td>
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<td>&gt;25 h</td>
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<td>NA</td>
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<td>3–25 h</td>
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<td>&lt;3 h</td>
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<td>C&lt;sub&gt;max&lt;/sub&gt;/MIC</td>
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<td>&gt;8</td>
<td>0/8</td>
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<td>1–8</td>
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<td>&lt;1</td>
<td>0/3</td>
<td>NA</td>
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<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;/MPC</td>
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<tr>
<td>&gt;1.2</td>
<td>0/8</td>
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<tr>
<td>0.2–1.2</td>
<td>11/13</td>
<td>.018</td>
</tr>
<tr>
<td>&lt;0.2</td>
<td>1/4</td>
<td>.571</td>
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<tr>
<td>Time above the MPC</td>
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<tr>
<td>&gt;20%</td>
<td>0/8</td>
<td>NA</td>
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<tr>
<td>&lt;20%</td>
<td>12/17</td>
<td>.049</td>
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**NOTE.** Indices were calculated using total drug concentrations. A reduction in AUC<sub>24</sub>/MIC (where AUC<sub>24</sub> is the area under the drug concentration time curve in a 24-h interval) and AUC<sub>24</sub>/mutant prevention concentration (MPC) of >30% was observed when free drug concentrations were used, with little impact on the fraction of rabbits with resistant bacteria in each category. C<sub>max</sub> maximum concentration; NA, not applicable.

<sup>a</sup> Time in the window (t<sub>msw</sub>) is not presented in the table because it fell into 2 categories: t<sub>msw</sub> > 33% correlated with selection of resistance (9/9 rabbits; P = .006) when C<sub>msw</sub>/MPC. The same index changed to >80% when C<sub>msw</sub>/MPC (2/2 rabbits; P = .1).

<sup>b</sup> P values were calculated by Fisher’s exact test, with a set of 3 infected but untreated rabbits used as a control. High values indicate no difference with the control.
fluctuating fluoroquinolone concentrations with respect to defining selection window boundaries for in vitro models [28–30]; the data in figure 2 demonstrate that agar plate determinations of MIC<sub>c</sub> and MPC fit well with the boundaries of the selection window seen in vivo at the site of infection. Thus, agar plate data appear to be predictive for fluoroquinolones.

Because MPC is a bacteriostatic threshold whereas levofloxacin is a bactericidal agent, a correction is required when concentrations are high enough to allow killing of first-step resistant mutant subpopulations (such was probably the case in an earlier rabbit study in which serum levels of moxifloxacin needed to exceed the MPC for only half the dosing period to restrict the recovery of pneumococcal mutants in the lungs [15]). Corrections involving fluctuating drug concentrations are generally made by use of PK/PD indices that estimate drug exposure [24]. For example, AUC<sub>24</sub>/MIC can be used empirically to predict favorable outcome in patients when susceptible populations are considered [25, 31]. Therefore, AUC<sub>24</sub>/MIC can be used to define the bottom of the selection window. For the top of the window, we apply the pharmacodynamic idea to resistant mutant subpopulations. Then AUC<sub>24</sub>/MPC is the appropriate parameter, because the MPC is the MIC of the least-susceptible single-step mutant [9]. An in vitro study in which Escherichia coli was treated with ciprofloxacin also argues for the use of AUC<sub>24</sub>/MPC. In that study, AUC<sub>24</sub>/MPC = 22 h correlated with restricted outgrowth of resistant mutant subpopulations [32]. In the present work, the mutant restrictive value of AUC<sub>24</sub>/MPC was 25 h, using total drug concentrations for the calculation. Correction for protein binding of levofloxacin, which was 30% in the present perforated-plastic-ball system, lowered the in vivo AUC<sub>24</sub>/MPC threshold to 18 h. Thus, the fit between the in vitro ciprofloxacin–E. coli data and the in vivo levofloxacin–S. aureus determinations is good (in vivo systems are expected to have lower values when host defense systems eliminate resistant mutants).

Other pharmacodynamic indices—such as C<sub>MIC</sub>/MIC, C<sub>MIC</sub>/MPC, time above the MPC, and time in the window—also showed a statistically significant correlation with the selection of resistance. The present work did not identify the most-predictive index, because only 1 dosing frequency was tested (multiple dosage administration and frequency combinations must be tested to identify the most-predictive index). AUC<sub>24</sub>/MIC and C<sub>MIC</sub>/MIC have been traditionally used for predicting treatment outcome and resistance [8, 25, 33]. However, for identifying mutant-restrictive conditions, use of MIC-based indices relies on the assumption that the MIC is proportional to the MPC, which is, at best, poorly supported [34–36].

Keeping antimicrobial concentrations above the MPC or AUC<sub>24</sub>/MPC >25 h (18 h when free drug is considered) is a straightforward way to restrict the acquisition of resistance. However, the acquisition of resistant mutants by placing concentrations inside the window is complex. For example, in an in vitro model, conditions at the center of the window were suitable for selecting a double mutant, which caused the least-susceptible mutant to be recovered at the middle rather than at the top of the window [30]. In the present rabbit/perforated-plastic-ball system, the drug concentration needed to be inside the window for ~80% of the time to enrich mutants when those concentrations fluctuated above and below the MPC. When the concentrations fluctuated above and below the MIC<sub>c</sub>, they needed to be inside the window for only 33% of the time to enrich mutants (table 2). This difference probably derives from more-abundant preexisting resistant mutant subpopulations being able to survive and proliferate near the bottom of the window [23] and from the killing of some mutants when drug concentrations are close to the top of the window.

In conclusion, the data in figure 2 demonstrate that agar plate determinations fit the mutant selection window for fluoroquinolone treatment of rabbits infected with S. aureus. Because agar plate assays are routine in clinical laboratories, implementation of selection window dosing strategies is feasible. The next steps for the tissue-cage model are to use more virulent strains and to allow bacterial populations to reach 1 x 10<sup>9</sup> cells by in vivo growth from a smaller inoculum. The latter may be difficult with S. aureus, because high bacterial density achieved by long periods of in vivo growth tends to make tissue-cage fluid very viscous and difficult to sample.

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