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Influence of inoculum size on the selection of resistance in *Escherichia coli* by quinolone in a mouse-thigh bacterial infection model : a PK/PD analysis

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ABSTRACT

Maintaining quinolone concentrations outside the Mutant Selection Window (MSW) between the MIC and Mutant Prevention Concentration (MPC) was suggested by *in vitro* and *in vivo* studies to prevent the selection of resistant mutants. However, selection may also depend on the presence of resistant bacterial mutants at the start of treatment that is highly dependent on the initial inoculum size. In this study, a mouse-thigh bacterial infection model was used to test the influence of different exposures to marbofloxacin on the selection of resistant bacteria after infection with a low (10^5 CFU) or high (10^8 CFU) initial inoculum of *Escherichia coli*. The inoculum size was shown to influence the exposure to marbofloxacin and the values of PK/PD indices. When the abilities of the indices time within the MSW (T_{MSW}), AUC_{24}/MIC and C_{max}/MIC to predict the selection of resistant bacteria were compared, only the T_{MSW} appeared to be a good predictor of the prevention of resistance for values less than 30%. When the T_{MSW} was higher than 34%, the selection of resistant bacteria occurred less often in thighs initially infected with the low inoculum (11/24, 46 %) than in those infected with the high inoculum (30/36, 80 %) suggesting that the selection of resistant mutants may depend on both the T_{MSW} and inoculum size. The relevance of these results merits further investigation to test different strategies of antibiotic therapy depending on the expected bacterial burden at the infectious site.

32 **INTRODUCTION**

33 Resistances to fluoroquinolones can occur spontaneously in bacterial populations at a
34 frequency of about 10^{-6} to 10^{-8} (4) following a stepwise process that involves mutations in
35 genes coding for the targets, DNA gyrase and topoisomerase IV (22, 28). Consequently, if the
36 bacterial load at the infectious site exceeds the inverse of the mutation frequency, it can be
37 presumed that a small resistant subpopulation already coexists with a larger susceptible
38 population, before any antimicrobial treatment is administered. Traditionally, *in vitro*
39 antimicrobial studies and animal infection models have been used to assess the reduction in
40 the total bacterial population at an infectious site while often ignoring the impact of drug
41 pressure on amplification of the drug-resistant subpopulation (7). Thus, the values of
42 pharmacokinetic/pharmacodynamic indices determined from these experiments were
43 previously selected to predict the bacteria killing and not the selection of resistant bacteria.
44 These PK/PD indices : *f*AUC/MIC (area under the free-plasma concentration curve divided by
45 the Minimal Inhibitory Concentration (MIC), fC_{max}/MIC (peak free-plasma concentration
46 divided by the MIC) and $fT_{>MIC}$ (time the free concentrations are above the MIC), are all
47 expressed as a function of the MIC, which is the pharmacodynamic parameter used to
48 describe the susceptibility of the major drug-susceptible population. From experiments carried
49 out with fluoroquinolones, the Mutant Prevention Concentration (MPC) has been proposed to
50 assess the susceptibility of the resistant subpopulation (11, 23, 29). MIC and MPC then define
51 the boundaries of the so-called Mutant Selection Window (MSW), which is the range of
52 antibiotic concentrations that would favour the selection of first-step mutants (29). First-step
53 mutants of *Staphylococcus aureus* and *Streptococcus pneumoniae* were selected by
54 ciprofloxacin, levofloxacin or moxifloxacin when antibiotic concentrations fell within the
55 MSW *in vitro* (5, 6, 16, 30). A study carried out in a rabbit lung infection model with
56 *Streptococcus pneumoniae* showed that the selection of resistant bacteria occurred

57 systematically when concentrations of gatifloxacin were within the MSW (T_{MSW}) for more
58 than 45% of the treatment duration (9). Another experiment in rabbits infected by
59 *Staphylococcus aureus* also showed that drug concentrations needed to be at the bottom of the
60 MSW (just above the MIC) for only 33 % of the time to enrich mutants (10). These *in vivo*
61 results, which suggest that fluoroquinolone concentrations need to be outside the MSW for
62 most of the time to prevent the selection of resistant mutants, were observed with a high
63 inoculum size (more than 10^9 CFU). However, the bacterial burdens to be eradicated at the
64 infectious site may be very low or null when antibiotics are used preventively as in
65 prophylaxis of gram-negative bacteremia in immuno-compromised patients (24) or
66 metaphylaxis in veterinary medicine. For both human and veterinary medicine, prophylaxis is
67 the administration of antimicrobials to exposed individuals considered at risk, but before the
68 expected onset of disease. Metaphylaxis consists of treating all animals at the herd level when
69 there is a clinical disease in only some animals (26). In the case of prophylaxis and
70 metaphylaxis, the bacterial inocula at the beginning of the treatment are presumably much
71 smaller than those targeted when patients express clinical signs or are critically ill and
72 therefore the likelihood of a mutant appearing may be low, particularly if the bacterial load is
73 less than the inverse of the mutation rate.

74 We previously observed *in vitro* that emergence of resistance was more frequent when
75 both the time within MSW (T_{MSW}) and the bacterial inoculum size increased (14) and the aim
76 of this study was to test, *in vivo,* the ability of T_{MSW} and also of AUC_{24}/MIC and C_{max}/MIC
77 indices to predict the selection of resistant bacteria in a low and a high inoculum. We assessed
78 the selection of a pre-existing resistant subpopulation of *Escherichia coli* in mouse thighs
79 infected with the two bacterial inoculum sizes, after different exposures to marbofloxacin, a
80 fluoroquinolone extensively used in veterinary medicine.

81 **MATERIALS AND METHODS.**

82 **Bacteria and antibiotic.**

83 *Escherichia coli* ATCC25922 was used as the susceptible bacteria and an *Escherichia coli*
84 isolated from a previous thigh infected by *E. coli* ATCC25922, treated with marbofloxacin
85 and carrying a S83L mutation on GyrA was used as the resistant bacteria. Marbofloxacin, a
86 third generation quinolone, was kindly provided by Vetoquinol, Lure, France.

87 **In vitro susceptibility testing. (i) MIC determination.**

88 MICs were determined in triplicate for the susceptible and mutant bacteria by a broth
89 microdilution method according to CLSI reference methods.

90 **(ii) MPC determination.** The MPC was determined as previously described (4). Briefly, an
91 overnight culture of the tested bacteria in Mueller Hinton broth (MHB) was concentrated 100
92 times in NaCl 0.9% to obtain a suspension containing 10^{10} CFU/mL. One hundred microliters
93 of this suspension were then plated on MH agar containing various concentrations of
94 marbofloxacin obtained by successive two-fold dilutions. The MPC was the lowest
95 marbofloxacin concentration preventing the growth of bacterial colonies after incubation for
96 72 hours at 37°C. Determinations were done in triplicate.

97 **PCR amplification of quinolone resistance-determining regions (QRDRs) and DNA**
98 **sequencing.**

99 Genomic DNA was isolated from bacterial strains and used as the template in PCR
100 amplification of the QRDRs of gyrA and parC genes. PCR amplification and nucleotide
101 sequencing were carried out with previously described primers (13).

102 **Inoculum preparation.**

103 A few colonies from an overnight culture of susceptible (ATCC25922) and resistant (mutant)
104 *Escherichia coli* were grown at 37°C for 18 h in 50 mL of MHB and in 10 mL of MHB
105 supplemented with 0.128 µg/mL marbofloxacin (the first concentration below the MIC of the

106 resistant bacteria) respectively. Bacteria were then collected by centrifugation at 3000 g for 10
107 min and resuspended in NaCl 0.9% to obtain a final suspension containing 10^9 CFU/mL of
108 susceptible bacteria and 10^3 CFU/mL of resistant bacteria. This suspension, which
109 corresponded to the high inoculum, was then diluted 1000 times to obtain the low inoculum.
110 This method to prepare the bacterial inoculum implied that the low inoculum (6.6±0.21 $\log_{10}$
111 CFU/mL) may have contained resistant mutants in about 40% of the experiments depending
112 on the sampling.

113 **Animals.**

114 Female Swiss mice (Charles River Laboratories, L'arbresle, France) were used for all studies.
115 All animal procedures were conducted in accordance with accepted human standards of
116 animal care under the agreement number A 31909 for animal experimentation from the
117 French Ministry of Agriculture.

118 **Neutropenic mouse thigh infection model.**

119 The model used was previously described by Andes and Craig (2). Briefy, female Swiss mice
120 were made neutropenic by intraperitoneal injection of cyclophosphamide (Sigma-Aldrich,
121 Saint Quentin Fallavier, France) at rates of 150 mg/kg and 100 mg/kg of body weight on days
122 1 and 4 respectively. On day 5, the mice were infected by intramuscular injection of 100 µL
123 of the high or low inoculum in each thigh (six thighs per dose, dosing schedule and inoculum
124 size). Marbofloxacin was administered intraperitonally beginning 2 hours after infection. The
125 total doses were 0, 2, 5, 10 and 20 mg/kg marbofloxacin for mice sacrificed at 24 hours and 0,
126 4, 10, 20 and 40 mg/kg marbofloxacin for mice sacrificed at 48 hours after infection. For each
127 dose of marbofloxacin, one group of mice received the total dose in a single administration
128 two hours after infection and another group received the same total dose divided into two or
129 four administrations for mice sacrificed 24 or 48 hours respectively after infection (that
130 corresponded to an administration twice a day). The mice were sacrificed by an

131 intraperitoneal injection of pentobarbital (DolethalND, Vetoquinol, France) 2 hours (non-
132 infected mice), 24 or 48 hours after the infection. Both thighs were aseptically removed and
133 homogenized in 10 mL of NaCl 0.9%. The homogenates were centrifuged at 3000 g for 10
134 minutes and washed twice in 10 mL of NaCl 0.9%. Ten microliters of successive 10-fold
135 dilutions of homogenates were then plated in triplicate on MH drug-free agar plates or on MH
136 plates supplemented with 0.128 µg/mL of marbofloxacin. The colonies were counted after
137 overnight incubation at 37°C. If the bacterial counts were less than 1000 CFU/thigh, 100 µL
138 of the homogenates were plated on agar. If the colonies were too small, incubation was
139 continued for a further 24 hours. The lowest level of detection was 100 CFU/thigh and
140 bacteria were considered eradicated below this level.

141 **Pharmacokinetics.**

142 Female Swiss mice, satellites from the pharmacodynamic assay, were treated by
143 cyclophosphamide as described above. On the fifth day, the mice were infected with either the
144 high or low inoculum in each thigh and two hours later were administered a single
145 intraperitoneal dose of marbofloxacin. The doses tested were 1 and 5 mg/kg marbofloxacin
146 for the low inoculum and 5 and 20 mg/kg marbofloxacin for the high inoculum. Groups of 3
147 or 4 mice each were anesthetized by intraperitoneal injection of pentobarbital (DolethalND,
148 Vetoquinol, France) 0.25, 0.5, 1, 2, 4, 6, 8 or 24 hours after dosing. Blood samples (one
149 sample from each animal) were collected by puncture of the caudal vena cava. Protein binding
150 was assessed by ultrafiltration as previously described (20). Briefly, plasma was spiked with
151 0.05, 0.1, 0.5, 1, 5 or 10 µg/mL marbofloxacin and was incubated for 30 min at 37°C. Plasma
152 was then transferred to Centrifree devices (Millipore Corporation, Billerica, MA, USA) and
153 centrifuged at ambient temperature for 30 min at 1200 g. The volumes of the ultrafiltrates
154 were at least 200 µL. A simple and sensitive high performance liquid chromatography
155 method, using ultraviolet detection at 295 nm to determine the marbofloxacin concentrations

156 in plasma and ultrafiltrates, was adapted from Schneider *et al.* (27). Briefly, samples were
157 extracted by solid phase extraction on a C8 100 mg cartridge. Marbofloxacin and the internal
158 standard, ofloxacin, were separated on a reverse-phase C18 Inertsil ODS-3 column and eluted
159 with 25 mM citrate buffer (pH=3.0) and acetonitrile in an 85:15 ratio. The standard
160 calibration curve for marbofloxacin, using a weighted linear regression model, was linear for
161 concentrations ranging from 0.01 to 2 µg/mL. The intra-day and inter-day precision ranged
162 from 4.58 to 9.27% and from 4.85 to 9.46% respectively. The accuracy varied from 91 to
163 108%.

164 **PK-PD analysis.**

165 The data obtained with a single intraperitoneal marbofloxacin injection of 5 mg/kg were
166 analysed separately for each inoculum size, using WinNonlin version 5.2 (Pharsight
167 Corporation, Mountain View, CA, USA). For the two inocula tested (low and high), a naïve
168 pooling approach was used to fit the marbofloxacin data with a bi-exponential model. The
169 AUC_{tot}, AUC_{24} and AUC_{48} were calculated by integrating the equation used to fit the data
170 from zero to infinity, from zero to 24 hours and from zero to 48 hours, respectively (17). The
171 terminal half-life ($T_{1/2elim}$) was calculated as the naperian logarithm of 2 divided by the
172 elimination rate constant. Dose-proportionality was assessed from the kinetic data obtained
173 with 1 mg/kg marbofloxacin for the low and with 20 mg/kg marbofloxacin for the high
174 inoculum. Marbofloxacin pharmacokinetic parameters obtained from mice treated with 5
175 mg/kg were then used to estimate the T_{MSW}, AUC_{24}/MIC and C_{max}/MIC for each tested
176 marbofloxacin dosing regimen (single and fractionated doses) and bacterial inoculum size.
177 The AUC_{24}/MIC for mice sacrificed 48 hours after the infection were determined by dividing
178 the AUC_{48}/MIC by 2 to obtain a mean AUC_{24}/MIC for each day.

179 The sigmoid inhibitory E_{max} models describing the dose-response relationships for the low and
180 the high inoculum after 24 hours of exposure to marbofloxacin were delineated with
181 WinNonlin version 5.2 (Pharsight Corporation, Mountain View, CA, USA).

182 **RESULTS**

183 **Susceptibility studies.** The MICs of marbofloxacin were 0.008 and 0.256 µg/mL for the
184 susceptible and mutant strains of *Escherichia coli* respectively. The marbofloxacin MPC was
185 0.256 µg/mL for the susceptible bacteria and corresponded to the MIC of the mutant strain.
186 The Mutant Selection Window (MSW) for the susceptible bacteria was therefore the range of
187 marbofloxacin concentrations between the MIC (0.008 µg/mL) and the MPC (0.256 µg/mL).
188 The term "resistant bacteria" in the present paper should be understood as bacteria growing on
189 0.128 µg/mL marbofloxacin.

190 **Pharmacokinetics.** The observed (total) and the predicted (total) plasma concentrations of
191 marbofloxacin following single intraperitoneal doses of 1 and 5 mg/kg in neutropenic mice
192 infected with the low inoculum and doses of 5 and 20 mg/kg in neutropenic mice infected
193 with the high inoculum are shown in Figure 1. The values for the marbofloxacin
194 pharmacokinetic parameters obtained for each dose are shown in Table 1. Marbofloxacin
195 kinetics in mice differed considerably between the two inoculum sizes, the AUC (exposure) in
196 mice subjected to the high inoculum being two times greater after the same dose of 5 mg/kg.
197 Dose-proportionality was assessed with marbofloxacin doses of 1 mg/kg for the low and 20
198 mg/kg for the high inoculum, respectively. The dose/AUC ratios with both doses were similar
199 to those of mice infected with the same inoculum size and treated with 5 mg/kg
200 marbofloxacin, suggesting dose-proportionality for the range of concentrations tested. The
201 binding of marbofloxacin to mice plasma proteins, between 0.05 µg/mL and 10 µg/mL, was
202 less than 10 % and the PK/PD indices were determined from the total plasma concentrations.

203 The time within the MSW (T_{MSW}), and the AUC_{24}/MIC and C_{max}/MIC values, obtained after
204 the administration of each marbofloxacin dosing regimen to mice sacrificed 48 hours after
205 infection with the low or high inoculum, are presented in Table 2. Assuming dose-
206 proportionality, the calculated AUC_{24} values were directly proportional to the total dose of

207 marbofloxacin for a given inoculum size and were independent of dose fractionation. The
208 C_{max} values were also linked to the total dose but the values were four times less after
209 fractionation of the doses into four administrations (twice a day for 2 days) than after the
210 administration of single doses. The influence of marbofloxacin dosage regimen (both total
211 dose and fractionation) and inoculum size on the T_{MSW} indice was much more complex and
212 resulted in an overlap of T_{MSW} values between the dosage regimens.

213 **PK-PD analysis.**

214 **(i) total bacterial populations.** The total bacterial populations after 24 hours of exposure to
215 marbofloxacin are shown for each inoculum size and each dosing regimen in Table 3. The
216 lowest tested dose of 2 mg/kg marbofloxacin, corresponding to AUC_{24}/MIC values of 175 and
217 387 h for the low and high inoculum respectively, was bacteriostatic. The dose of 5 mg/kg
218 marbofloxacin, corresponding to AUC_{24}/MIC values of 450 and 962 h for the low and high
219 inoculum respectively, reduced the bacterial population by more than one log for both
220 inocula. Figure 2 shows that the dose-response relationship was best described by inhibitory
221 E_{max} curves for the low (equation1) and the high inoculum (equation 2).

222 Log_{10} CFU/thigh = 9.58-[(6.28*dose)/(dose+1.27)]

223 Log_{10} CFU/thigh = 10.02-[(5.70*dose)/(dose+3.92)]

224 By assuming dose-proportionality for a given inoculum, the relationships between the dose or
225 AUC_{24}/MIC and the reduction of the total bacterial population had exactly the same shape.
226 After 24 hours, we observed the same reduction in the total bacterial population whatever the
227 dose fractionation even though C_{max} were four times lower and $T_{>MIC}$ always longer after
228 fractionated doses than after single doses. As a consequence, no relationship could be found
229 between C_{max}/MIC or $T_{>MIC}$ and the reduction of the total bacterial population.

230 The bacterial populations after 48 hours of exposure to marbofloxacin are shown in Table 4
231 for each inoculum size and each dosing regimen. All control mice (dose 0) and all mice

232 infected by the high inoculum and treated with a total dose of 4 mg/kg marbofloxacin,
233 corresponding to AUC_{24}/MIC and AUC_{24}/MPC values of 387 and 12 h respectively, were
234 dead within 48 hours post-inoculation. At the higher doses and also for the low inoculum, the
235 bacterial population was eradicated in some thighs, the frequency of eradication being greater
236 the higher the total dose. However, no clear relationship between reduction of the bacterial
237 populations after 48 hours of exposure to marbofloxacin and the total dose or AUC_{24}/MIC
238 was apparent. After single doses associated with higher C_{max}/MIC, the bacterial populations
239 were lower when the initial inoculum was low but higher when the initial inoculum was high
240 than after the same fractionated doses. This strongly suggested that there was no relationship
241 between C_{max}/MIC and the reduction of the total bacterial population.
242 **(ii) resistant bacterial populations.** When the treatment was started, no resistant bacteria
243 were detected in mice infected with the low inoculum while 2.74±0.41 log_{10} CFU of resistant
244 bacteria were present in mice infected with the high inoculum. Twenty-four hours after
245 infection, no resistant bacteria were recovered from the control mice infected with the low
246 inoculum. The proportions of resistant bacteria after 24 hours and 48 hours versus dose are
247 shown in Figure 3. After 24 hours, with the low inoculum, resistant bacteria in a proportion of
248 3.10^{-2} of the total bacteria count were only found in one thigh of a mouse treated with the
249 fractionated dose of 2 mg/kg marbofloxacin (Figure 3A). With the high inoculum, the
250 proportion of resistant bacteria in all thighs was always greater than 10^{-5} except for four thighs
251 of mice treated with a total dose of 20 mg/kg marbofloxacin in which all resistant bacteria had
252 been eradicated (Figure 3C).
253 After 48 hours, no resistant bacteria were detected in mice infected with the low inoculum and
254 treated with single doses of marbofloxacin whereas resistant bacteria were recovered in 46 %
255 of the thighs treated with fractionated doses. The proportion of resistant bacteria ranged from
256 10^{-2} to 1 in these thighs (Figure 3B). For the high inoculum, resistant bacteria were recovered

257 in all thighs after single doses whereas resistant bacteria were only recovered in 33 % of the
258 thighs treated with fractionated doses. The proportions of resistant bacteria were always
259 greater than 10^{-2} after single doses and ranged from 10^{-6} to 10^{-2} after the administration of
260 fractionated doses (Figure 3D). As these results indicated a net difference in the selection of
261 resistant bacteria after administrations of single or fractionated doses, it can be presumed that
262 no simple relationship existed between the total dose and the selection of resistant bacteria.
263 We then investigated the ability of T_{MSW} and also AUC_{24}/MIC and C_{max}/MIC indices, that
264 reflected the overall exposure, to predict the selection of resistant bacteria. The proportions of
265 resistant bacteria after 48 hours of treatment are shown against the PK/PD indices in Figure 4.
266 The relationship between the proportion of resistant bacteria and the AUC_{24}/MIC and
267 C_{max}/MIC indices was poor whereas a clear relationship existed between T_{MSW} and the
268 prevention of resistance. Indeed, after 48 hours of treatment, the marbofloxacin dosing
269 regimen associated with a T_{MSW} of 34 to 82 % led to the selection of resistant bacteria in 46 %
270 of the thighs with the low inoculum and in 80% with the high inoculum. By contrast no
271 selection of resistant bacteria occurred when T_{MSW} was less than 30 %, whatever the inoculum
272 size.

273 **DISCUSSION**

274 Maintaining drug concentrations outside the MSW, which is the range of antibiotic
275 concentrations favouring the selection of resistant mutants, has been suggested to restrict
276 mutant outgrowth (12, 29). Previous *in vivo* studies that showed an enrichment of mutants
277 when drug concentrations were most of the time within the MSW tested only one inoculum
278 size (1, 8-10, 18). However, the bacterial load at the site of infection, against which antibiotic
279 drugs are prescribed, can vary considerably whether the treatment is prophylactic or curative.
280 The aim of the present study was to investigate the ability of T_{MSW} and also of AUC_{24}/MIC
281 and C_{max}/MIC indices to predict the selection of resistant bacteria in a mouse-thigh infection
282 model. Mice infected with a low or high inoculum of *Escherichia coli,* already containing a
283 proportion of 10^{-6} resistant mutants, were treated with different regimens of marbofloxacin to
284 assess the influence of dose, actual exposure (as determined by the AUC_{24}/MIC, C_{max}/MIC
285 and T_{MSW}) and inoculum size on the selection of resistant bacteria.

286 Pharmacokinetic studies in which 5 mg/kg marbofloxacin was administered to infected
287 animals revealed a large difference between the rates of elimination of marbofloxacin with the
288 two tested inoculum sizes. Higher elimination half-lives and decreased systemic clearance
289 were previously observed in infected calves (20) and horses (25) that were treated with
290 marbofloxacin. Explanations for alteration of the drug elimination pathways may be the
291 production by gram-negative bacteria of endotoxins that were shown to decrease the activity
292 of hepatic metabolism, the renal blood flow and glomerular filtration rate (19).

293 Whatever the regimen, a given total dose of marbofloxacin produced a similar
294 reduction in bacterial population size after 24 hours of treatment, thereby suggesting that the
295 AUC_{24}/MIC indice, which was proportional to the total dose, was an appropriate predictor of
296 bacterial killing. In addition, after 48 hours of exposure to marbofloxacin, the only PK/PD
297 indice that was identical for all mice that died after infection with the high inoculum and a

298 treatment with 4 mg/kg marbofloxacin for 48 hours, was also the AUC_{24}/MIC since the
299 C_{max}/MIC and T_{MSW} indices differed between mice treated with single doses and mice treated
300 with fractionated doses. In a previous study, an AUC/MIC value of 70 h for gatifloxacin was
301 suggested to have a bacteriostatic effect in a thigh infection of *Klebsiella pneumoniae* in
302 neutropenic mice (3). In our study, the value of AUC_{24}/MPC (corresponding to the
303 AUC_{24}/MIC for the resistant bacteria) was 12.1 h for a dose of 4 mg/kg marbofloxacin, which
304 would explain the inability of this dose to slow the growth of the resistant subpopulation
305 enough to prevent death. For the susceptible bacteria, the lowest AUC_{24}/MIC observed in our
306 study, i.e. 175 and 387 h for the low and high inoculum respectively, were always sufficient
307 to prevent bacterial growth for 24 hours. For the low inoculum, in which the initial resistant
308 population was assumed to be very small (the probability of the presence of at least one
309 resistant mutant was 0.4), an AUC/MIC value of 175 h was associated with the prevention of
310 the emergence of a resistant population for 24 hours in 11 thighs out of 12. This is in
311 agreement with the AUC/MIC value of 157 h found by Jumbe *et al.* (21) to prevent the
312 amplification of mutant strains in a large population of *Pseudomonas aeruginosa* treated with
313 levofloxacin. However, after 48 hours of treatment, the AUC_{24}/MIC became a poor predictor
314 of the selection of resistant bacteria. For example, no resistant bacteria were selected in the
315 low inoculum after a single dose of marbofloxacin corresponding to an AUC_{24}/MIC value of
316 175 h whereas resistant bacteria were selected in four thighs out of six after fractionated doses
317 corresponding to the same AUC_{24}/MIC value. With the high inoculum challenge, the opposite
318 was observed. Indeed, for the same AUC_{24}/MIC and AUC_{24}/MPC values of 3850 and 120 h
319 respectively, resistant bacteria were only selected after single doses. This could not be
320 explained by the PK/PD parameter C_{max}/MIC because the value of C_{max} that prevented the
321 selection of resistant bacteria after dose fractionation was 4 times lower than after a single
322 dose which did lead to the selection of resistant bacteria. So in our study the lack of

323 consistency in the selection of resistance, regarding inoculum size and the dosing regimen
324 after 48 hours of treatment, could not be explained by the AUC_{24}/MIC and C_{max}/MIC indices.
325 Indeed, no relationship was found in these cases between AUC_{24}/MIC or C_{max}/MIC and
326 prevention of the selection of resistant bacteria.
327 By contrast, when the marbofloxacin exposures expressed as T_{MSW} for each inoculum size
328 were examined, a T_{MSW} of less than 30% was found to predict the prevention of resistance
329 selection for both inoculum sizes. This result suggested that T_{MSW} could be a good predictor
330 of resistance prevention *in vivo* although a previous *in vitro* model with *Staphylococcus*
331 *aureus* showed no correlation between T_{MSW} and selection of resistant bacteria (5). However,
332 it was recently suggested that the discrepancies observed in the T_{MSW} ability to predict
333 selection may be explained by an additional influence of the position of antimicrobial
334 concentrations within the MSW on the selection of resistant bacteria (10, 15). Indeed, *in vivo*,
335 it was shown in a local infection of rabbits with *Staphylococcus aureus* that the T_{MSW} leading
336 to the emergence of resistance could be 30 % or 80 % depending on the area of the MSW in
337 which drug concentrations fluctuated (10). In our study, we found the same minimal time of
338 30 % associated with the selection of resistance. By relating marbofloxacin exposure to the
339 T_{MSW}, we clearly showed that all mice in which a selection of resistant bacteria occurred after
340 48 hours of marbofloxacin treatment were exposed to a T_{MSW} greater than 34 % for 48 hours
341 irrespective of the inoculum size. Similar results were also reported in a rabbit model of
342 human therapy with *Streptococcus pneumoniae* and moxifloxacin or gatifloxacin in which
343 selection occurred when the T_{MSW} ranged from 72.5 to 93.5% or when T_{MSW} was greater than
344 45% (8, 9). However, in these latter experiments, the rabbits were infected with a high
345 inoculum containing more than 10^9 CFU/lung. By testing 10^6 and 10^9 CFU/thigh we were able
346 to show that resistance was less frequently selected in the low inoculum than in the high
347 inoculum. After 48 hours of exposure to marbofloxacin, we showed that for a T_{MSW} of 34 %

348 or more, resistant bacteria were selected in 80 % (30/36) of the thighs exposed to the high
349 inoculum but in only 46 % of thighs (11/24) in mice infected by the low inoculum.
350 Interestingly, this percentage of 46 % corresponded to the probability of resistant bacteria
351 being present at the start of treatment with our method of inoculum preparation. The less
352 frequent selection of resistant bacteria in the low inoculum, which remained within the MSW
353 for a long time, seemed to be due to the fact that these bacteria were rarer from the start in the
354 low inoculum.

355 In conclusion, our results fully support the concept of MSW *in vivo* in a mouse-thigh
356 model since we showed that maintaining the antibiotic concentrations outside the MSW
357 prevented the selection of resistant mutants. We also showed in our pharmacokinetic study in
358 animals infected with either a high or low inoculum, that the size of the bacterial population at
359 the start of treatment could influence both the exposure to the antibiotic and the selection of
360 resistance. Indeed, the PK/PD study indicated that, for approximately the same T_{MSW}, the
361 selection of resistant bacteria occurred less often with the low inoculum than with the high
362 inoculum probably due to the less frequent presence of mutants in the low inoculum. Finally,
363 this study highlights the importance of assessing antibiotic exposure in critically ill patients or
364 animal models when establishing fluoroquinolone-dosing strategies in relation to the MSW
365 concept. The relevance of these results merits further investigation with pathogens of interest
366 and in a clinical context because of the common use of fluoroquinolones to treat a variety of
367 bacterial infections including urinary bacterial infection and to prevent febrile neutropenia in
368 cancer patients.

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372 **REFERENCES**

373 1. **Almeida, D., E. Nuermberger, S. Tyagi, W. R. Bishai, and J. Grosset.** 2007. *In*
374 *vivo* validation of the mutant selection window hypothesis with moxifloxacin in a
375 murine model of tuberculosis. Antimicrob Agents Chemother **51:**4261-6.

376 2. **Andes, D., and W. A. Craig.** 1998. In vivo activities of amoxicillin and amoxicillin-
377 clavulanate against *Streptococcus pneumoniae*: application to breakpoint
378 determinations. Antimicrob Agents Chemother **42:**2375-9.

379 3. **Andes, D., and W. A. Craig.** 2002. Pharmacodynamics of the new fluoroquinolone
380 gatifloxacin in murine thigh and lung infection models. Antimicrob Agents Chemother
381 **46:**1665-70.

382 4. **Blondeau, J. M., X. Zhao, G. Hansen, and K. Drlica.** 2001. Mutant prevention
383 concentrations of fluoroquinolones for clinical isolates of *Streptococcus pneumoniae.*
384 Antimicrob Agents Chemother **45:**433-8.

385 5. **Campion, J. J., P. Chung, P. J. McNamara, W. B. Titlow, and M. E. Evans.** 2005.
386 Pharmacodynamic modeling of the evolution of levofloxacin resistance in
387 *Staphylococcus aureus*. Antimicrob Agents Chemother **49:**2189-99.

388 6. **Campion, J. J., P. J. McNamara, and M. E. Evans.** 2004. Evolution of
389 ciprofloxacin-resistant *Staphylococcus aureus* in *in vitro* pharmacokinetic
390 environments. Antimicrob Agents Chemother **48:**4733-44.

391 7. **Craig, W. A.** 1998. Pharmacokinetic/pharmacodynamic parameters: rationale for
392 antibacterial dosing of mice and men. Clin Infect Dis **26:**1-10; quiz 11-2.

393 8. **Croisier, D., M. Etienne, E. Bergoin, P. E. Charles, C. Lequeu, L. Piroth, H.**
394 **Portier, and P. Chavanet.** 2004. Mutant selection window in levofloxacin and
395 moxifloxacin treatments of experimental pneumococcal pneumonia in a rabbit model
396 of human therapy. Antimicrob Agents Chemother **48:**1699-707.

397 9. **Croisier, D., M. Etienne, L. Piroth, E. Bergoin, C. Lequeu, H. Portier, and P.**
398 **Chavanet.** 2004. In vivo pharmacodynamic efficacy of gatifloxacin against
399 Streptococcus pneumoniae in an experimental model of pneumonia: impact of the low
400 levels of fluoroquinolone resistance on the enrichment of resistant mutants. J
401 Antimicrob Chemother **54:**640-7.

402 10. **Cui, J., Y. Liu, R. Wang, W. Tong, K. Drlica, and X. Zhao.** 2006. The mutant
403 selection window in rabbits infected with *Staphylococcus aureus*. J Infect Dis
404 **194:**1601-8.

405 11. **Dong, Y., X. Zhao, B. N. Kreiswirth, and K. Drlica.** 2000. Mutant prevention
406 concentration as a measure of antibiotic potency: studies with clinical isolates of
407 *Mycobacterium tuberculosis*. Antimicrob Agents Chemother **44:**2581-4.
408 12. **Drlica, K., and X. Zhao.** 2007. Mutant selection window hypothesis updated. Clin
409 Infect Dis **44:**681-8.
410 13. **Everett, M. J., Y. F. Jin, V. Ricci, and L. J. Piddock.** 1996. Contributions of
411 individual mechanisms to fluoroquinolone resistance in 36 *Escherichia coli* strains
412 isolated from humans and animals. Antimicrob Agents Chemother **40:**2380-6.
413 14. **Ferran, A., V. Dupouy, P. L. Toutain, and A. Bousquet-Melou.** 2007. Influence of
414 inoculum size on the selection of resistant mutants of *Escherichia coli* in relation to
415 mutant prevention concentrations of marbofloxacin. Antimicrob Agents Chemother
416 **51:**4163-6.
417 15. **Firsov, A. A., M. V. Smirnova, E. N. Strukova, S. N. Vostrov, Y. A. Portnoy, and**
418 **S. H. Zinner.** 2008. Enrichment of resistant *Staphylococcus aureus* at ciprofloxacin
419 concentrations simulated within the mutant selection window: bolus versus continuous
420 infusion. Int J Antimicrob Agents.
421 16. **Firsov, A. A., S. N. Vostrov, I. Y. Lubenko, K. Drlica, Y. A. Portnoy, and S. H.**
422 **Zinner.** 2003. In vitro pharmacodynamic evaluation of the mutant selection window
423 hypothesis using four fluoroquinolones against *Staphylococcus aureus.* Antimicrob
424 Agents Chemother **47:**1604-13.
425 17. **Gibaldi, M., and D. Perrier.** 1982. Pharmacokinetics, vol. 15. Marcel Dekker, Inc.
426 18. **Ginsburg, A. S., R. Sun, H. Calamita, C. P. Scott, W. R. Bishai, and J. H. Grosset.**
427 2005. Emergence of fluoroquinolone resistance in *Mycobacterium tuberculosis* during
428 continuously dosed moxifloxacin monotherapy in a mouse model. Antimicrob Agents
429 Chemother **49:**3977-9.
430 19. **Hasegawa, T., K. Takagi, and K. Kitaichi.** 1999. Effects of bacterial endotoxin on
431 drug pharmacokinetics. Nagoya J Med Sci **62:**11-28.
432 20. **Ismail, M., and Y. A. El-Kattan.** 2007. Comparative pharmacokinetics of
433 marbofloxacin in healthy and *Mannheimia haemolytica* infected calves. Res Vet Sci
434 **82:**398-404.
435 21. **Jumbe, N., A. Louie, R. Leary, W. Liu, M. R. Deziel, V. H. Tam, R. Bachhawat,**
436 **C. Freeman, J. B. Kahn, K. Bush, M. N. Dudley, M. H. Miller, and G. L.**
437 **Drusano.** 2003. Application of a mathematical model to prevent in vivo amplification
438 of antibiotic-resistant bacterial populations during therapy. J Clin Invest **112:**275-85.

439 22. **Khodursky, A. B., E. L. Zechiedrich, and N. R. Cozzarelli.** 1995. Topoisomerase
440 IV is a target of quinolones in *Escherichia coli*. Proc Natl Acad Sci U S A **92:**11801-
441 5.
442 23. **Marcusson, L. L., S. K. Olofsson, P. Komp Lindgren, O. Cars, and D. Hughes.**
443 2005. Mutant prevention concentrations of ciprofloxacin for urinary tract infection
444 isolates of *Escherichia coli.* J Antimicrob Chemother **55:**938-43.
445 24. **Moon, S., S. Williams, and M. Cullen.** 2006. Role of prophylactic antibiotics in the
446 prevention of infections after chemotherapy: a literature review. Support Cancer Ther
447 **3:**207-16.
448 25. **Peyrou, M., M. Y. Doucet, A. Vrins, D. Concordet, M. Schneider, and A.**
449 **Bousquet-Melou.** 2004. Population pharmacokinetics of marbofloxacin in horses:
450 preliminary analysis. J Vet Pharmacol Ther **27:**283-8.
451 26. **Phillips, I., M. Casewell, T. Cox, B. De Groot, C. Friis, R. Jones, C. Nightingale,**
452 **R. Preston, and J. Waddell.** 2004. Does the use of antibiotics in food animals pose a
453 risk to human health? A critical review of published data. J Antimicrob Chemother
454 **53:**28-52.
455 27. **Schneider, M., V. Thomas, B. Boisrame, and J. Deleforge.** 1996. Pharmacokinetics
456 of marbofloxacin in dogs after oral and parenteral administration. J Vet Pharmacol
457 Ther **19:**56-61.
458 28. **Sugino, A., C. L. Peebles, K. N. Kreuzer, and N. R. Cozzarelli.** 1977. Mechanism
459 of action of nalidixic acid: purification of *Escherichia coli* nalA gene product and its
460 relationship to DNA gyrase and a novel nicking-closing enzyme. Proc Natl Acad Sci
461 U S A **74:**4767-71.
462 29. **Zhao, X., and K. Drlica.** 2001. Restricting the selection of antibiotic-resistant
463 mutants: a general strategy derived from fluoroquinolone studies. Clin Infect Dis **33**
464 **Suppl 3:**S147-56.
465 30. **Zinner, S. H., I. Y. Lubenko, D. Gilbert, K. Simmons, X. Zhao, K. Drlica, and A.**
466 **A. Firsov.** 2003. Emergence of resistant *Streptococcus pneumoniae* in an *in vitro*
467 dynamic model that simulates moxifloxacin concentrations inside and outside the
468 mutant selection window: related changes in susceptibility, resistance frequency and
469 bacterial killing. J Antimicrob Chemother **52:**616-22.
470

471 **LEGENDS**

472 FIGURE 1 : Plasma marbofloxacin concentrations versus time after administration of a single
473 intraperitoneal dose of 1 (□) and 5 (○) mg/kg in mice infected with a low inoculum and doses
474 of 5 (●) and 20 (■) mg/kg in mice infected with a high inoculum. Fitted concentration-time
475 curves for the low (thin line) and the high (bold line) inoculum are also represented.

476

477 FIGURE 2 : Total bacterial population in each thigh infected with a low (○) or a high (■)
478 inoculum twenty-four hours after different total doses of marbofloxacin. Fitted curves for the
479 dose-effect relationship are shown for the low (dashed line) and the high (continuous line)
480 inocula. The bacterial populations were assumed to be 10 $\log_{10}$ CFU/thigh for the dead mice.

481

482 FIGURE 3 : Proportions of resistant bacteria in each thigh infected with a low (A-B) or a high
483 (C-D) inoculum twenty-four (A-C) or forty-eight (B-D) hours after different total doses of
484 marbofloxacin. Proportions obtained after single doses (●) and after fractionated doses (□) are
485 represented. Six thighs were tested per time, inoculum size and dosing regimen.

486

487 FIGURE 4 : Proportions of resistant bacteria in each thigh infected with low (circles) or high
488 (squares) inoculum after forty-eight hours of exposure to marbofloxacin versus AUC_{24}/MIC
489 (A), C_{max}/MIC (B) and T_{MSW} (C). Proportions are represented by empty symbols when they
490 were obtained after fractionated doses of marbofloxacin and by full symbols after single doses
491 of marbofloxacin.

492

493 TABLE 1.

494 Marbofloxacin pharmacokinetic parameters (elimination half-life ($T_{1/2elim}$), area under the
495 curve from 0 to infinity (AUC_{tot}), and the dose/AUC ratio) for mice infected with a low or a
496 high bacterial inoculum and treated with single intraperitoneal doses of 1, 5 or 20 mg/kg.

Inoculum size	**Low**		**High**	
Dose (mg/kg)	1	5	5	20
$T_{1/2elim}$ (h)	1.98	1.82	3.91	3.98
AUC_{tot} (µg.h.mL^{-1})	0.78	3.59	7.70	27.9
Dose/AUC (mL.h^{-1}.kg^{-1})	1282	1393	649	715

497

498 TABLE 2.

499 The estimated times within the Mutant Selection Window (T_{MSW}), AUC_{24}/MIC and C_{max}/MIC

500 values after administration of 4, 10, 20 or 40 mg/kg marbofloxacin for mice sacrificed after

501 48 hours of infection with the low or high inoculum. The values are given for each dosing

502 regimen (total dose administration in a single dose two hours after the infection or

503 fractionated twice a day).

	Low inoculum				**High inoculum**			
Total dose (mg/kg)	4	10	20	40	4	10	20	40
Fractionated Dose								
T_{MSW} (%)	57.7	66.6	65.4	59.4	87.4	63.8	33.8	10.9
AUC_{24}/MIC (h)	175	450	900	1800	387	962	1925	3850
C_{max}/MIC	95	237	475	950	75	187	375	750
Single Dose								
T_{MSW} (%)	18.9	19.7	19.8	19.8	42.3	42.5	42.5	42.4
AUC_{24}/MIC (h)	175	450	900	1800	387	962	1925	3850
C_{max}/MIC	375	950	1900	3800	300	750	1500	3000

504 The indices were estimated from kinetic studies performed in mice subjected to a low or high

505 inoculum and treated with 5mg/kg marbofloxacin.

506 TABLE 3.

507 Total bacterial populations (mean±SD) after 24 hours of exposure to different total doses of

508 marbofloxacin for the initial low and high inocula.

Total dose (mg/kg)	Start[a]	0	2	5	10	20
Low inoculum						
Single doses	6.15±0.48	9.52±0.35 *	6.17±1.12	4.36±1.36	4.31±1.45	4.06±0.83 (1)
Fractionated doses			5.07±1.62	4.77±1.16	4.51±1.07	3.56±1.19 (2)
High inoculum						
Single doses	8.65±0.17	**	8.66±0.61	6.86±0.58	6.39±1.37	5.54±1.28
Fractionated doses			7.86±0.61	6.03±0.77	6.21±0.55	4.71±0.93

509 Six thighs were tested for each inoculum, each dose and each dosing regimen. For start and at

510 dose 0 twelve thighs were tested per inoculum size. Numbers in brackets correspond to the

511 number of thighs in which bacteria were eradicated.

512 [a] Total bacterial population two hours after the time of infection

513 * one mouse out of six was dead

514 ** all six mice were dead

515 TABLE 4.

516 Total bacterial populations (mean±SD) after 48 hours of exposure to different total doses of

517 marbofloxacin administered in single or fractionated doses for initial low and high inocula.

Total dose (mg/kg)	0	4	10	20	40
Low inoculum					
Single doses	*	3.63±1.65	4.92±1.79	2.75±0.37	4.25±1.59 (4)
Fractionated doses	*	6.24±1.57 (2)	5.23±1.38 (3)	5.61±0.44 (4)	5.21±0.12 (4)
High inoculum					
Single doses	*	*	7.93±1.50	8.28±0.92	5.89±0.98
Fractionated doses	*	*	6.23±0.86	4.72±1.37	3.67±1.16 (1)

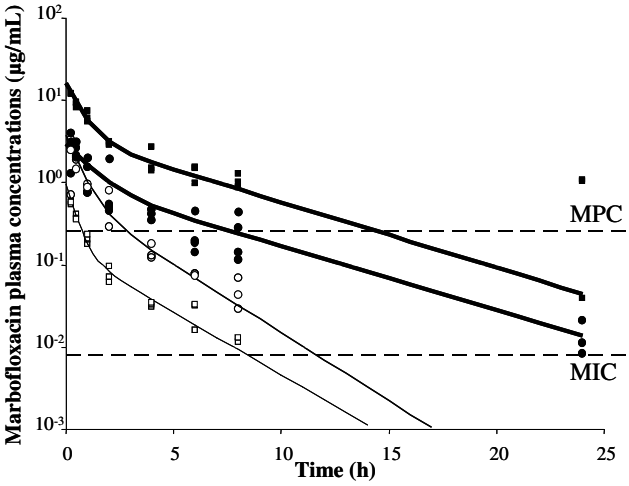
518 Six thighs were tested for each inoculum, each dose and each dosing regimen. Numbers in

519 brackets correspond to the number of thighs in which bacteria were eradicated. There was no

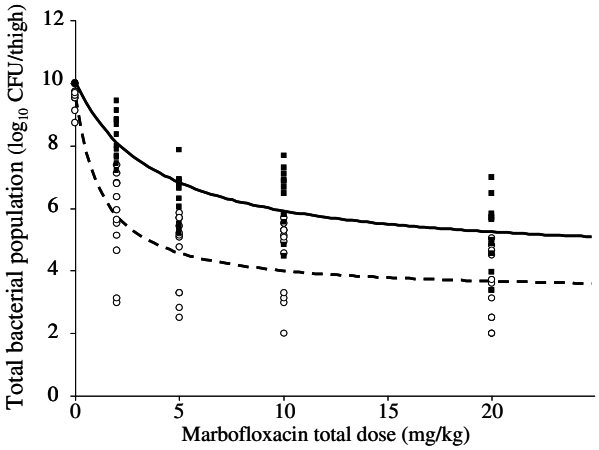
520 clear relationship between the dose and the reduction of the total bacterial population.

521 * all three mice were dead

522 FIGURE 1

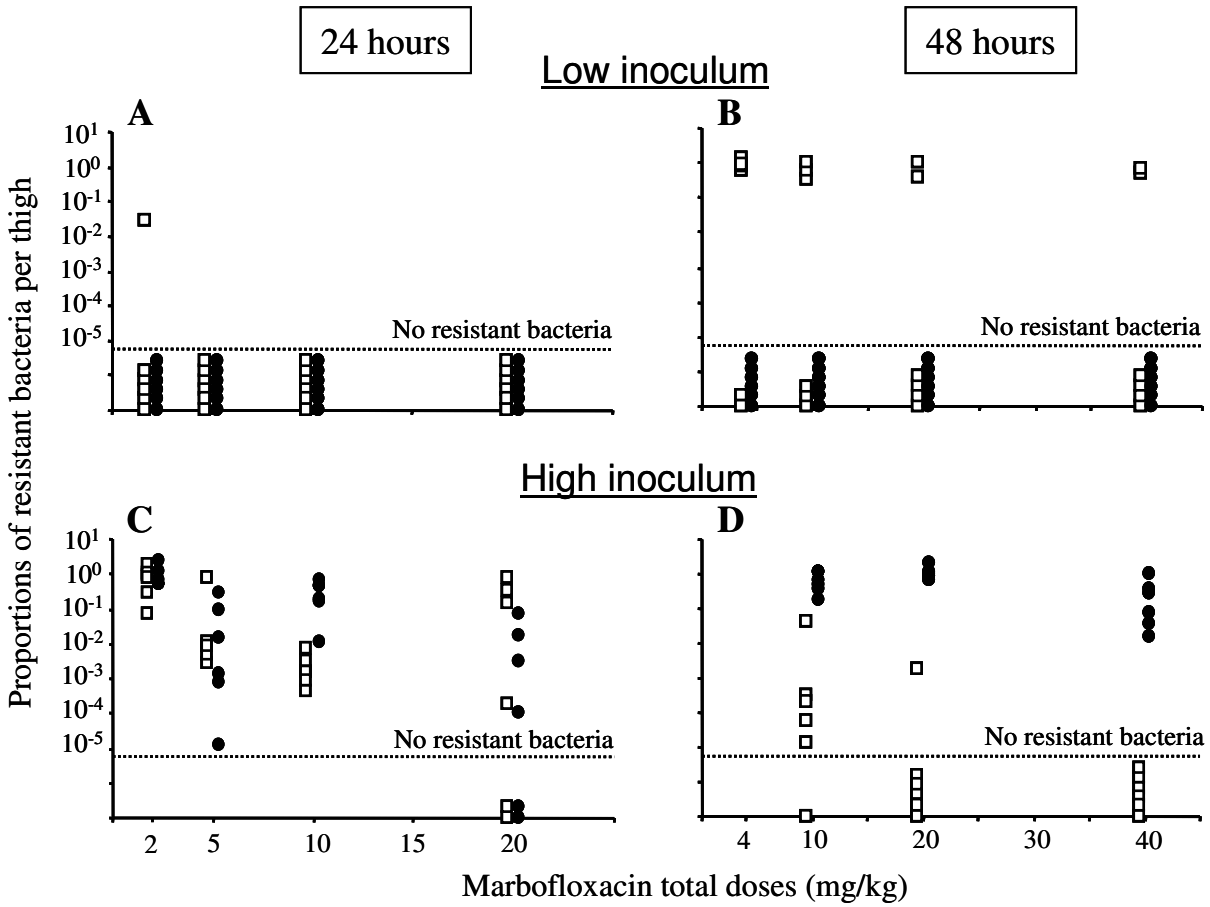

Marbofloxacin plasma concentrations (µg/mL)
10²
10¹
10⁰
10⁻¹
10⁻²
10⁻³
MPC
MIC
0
5
10
15
20
25
Time (h)

523 **FIGURE 2 :**

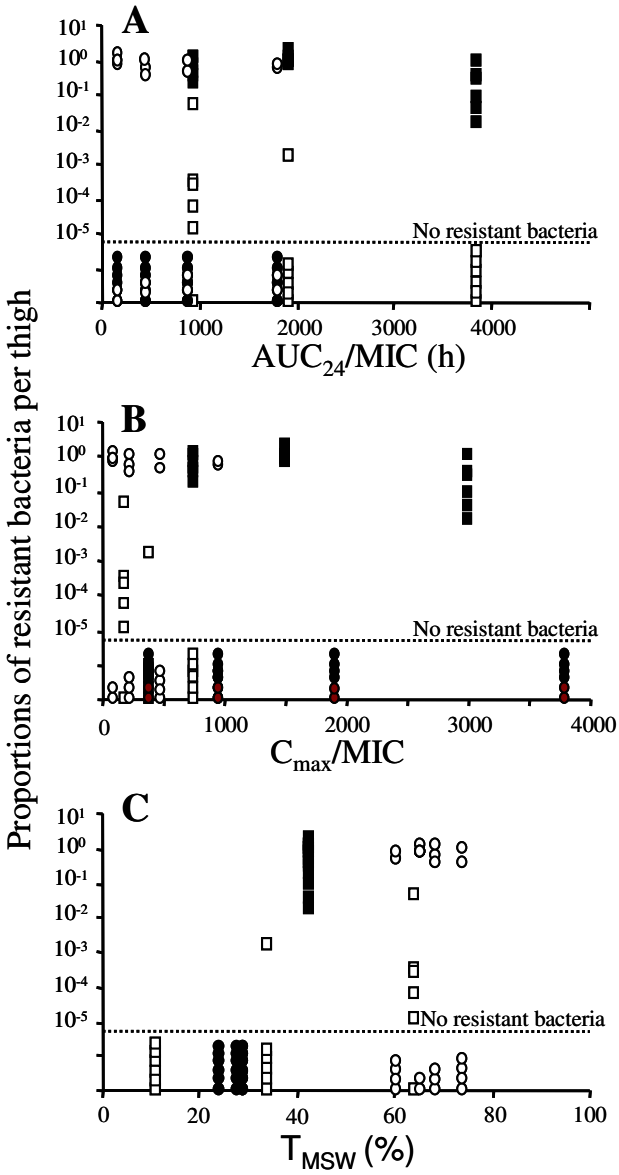

Total bacterial population (log10 CFU/thigh)
12
10
8
6
4
2
0
0
5
10
15
20
Marbofloxacin total dose (mg/kg)

524 FIGURE 3

525



526 FIGURE 4



527

528