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**Influence of Inoculum Size on the Selection of *Escherichia coli* Resistant Mutants in  
Relation to Mutant Preventive Concentrations of Marbofloxacin**

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16 We demonstrated using an *in vitro* pharmacodynamic model that the likelihood of selection of  
17 *Escherichia coli* resistant mutants to a fluoroquinolone was increased when the initial size of  
18 the bacterial population exposed to fluoroquinolone concentrations within the mutant  
19 selection window was increased

Resistant bacteria selected under the pressure of fluoroquinolone exposure expand from few spontaneously resistant mutant present before any treatment. When the bacterial load at the infectious site exceeds  $10^9$ - $10^{10}$  CFU (ref), it can be presumed if spontaneous mutation rate is about  $10^{-9}$ - $10^{-7}$  (Lindgren, AAC, 2003) that before any antibiotic treatment a small resistant subpopulation of first-step resistant mutants already coexists with the larger susceptible wild-type population.

Minimal Inhibitory Concentrations (MIC) allows the determination of the susceptibility of the major bacterial population and Mutant Prevention Concentration (MPC) the susceptibility of the small resistant subpopulation (2, 6, 10). MIC and MPC define the Mutant Selection Window (MSW), a range of antibiotic concentrations favouring the selection of first-step mutants (10). Previous studies (5, 11) have indicated that prevention of first-step mutants selection was obtained when fluoroquinolone concentrations exceeded the MPC for more than 80% of the dosage interval, i.e. when time within MSW ( $T_{MSW}$ ) was inferior to 20%. However, these studies only tested one inoculum size whereas the bacterial load increases during the time course of infections, and the likelihood of a mutant appearing may increase with inoculum size.

The aim of this study was to determine, using marbofloxacin a fluoroquinolone extensively used in veterinary medicine, an interaction between inoculum sizes ( $10^5$ ,  $10^7$  or  $10^9$  CFU/mL) and marbofloxacin exposures characterized by different  $T_{MSW}$  (0, 25 or 100%) on the selection of *Escherichia coli* resistant mutants.

Marbofloxacin MIC for *Escherichia coli* ATCC 25922 was determined by microdilution technique and MPC by a previously described method (1). MIC and MPC were 0.008 and 0.256  $\mu$ g/mL, respectively.

Bacteria suspended in Mueller-Hinton (MH) broth were exposed in an *in vitro* pharmacodynamic model to three monoexponential kinetic profiles of marbofloxacin to ensure  $T_{MSW}$  of 0, 25 and 100% corresponding to times above MPC ( $T_{>MPC}$ ) of 100, 75 and 0%. Bacteria exposures to marbofloxacin were determined from serial samples by HPLC and killing and regrowth of bacterial population were assessed by counting viable bacteria.

For all initial inoculum sizes, bacterial counts without antibiotic revealed similar exponential growth rate until the carrying capacity of the *in vitro* system was reached (about  $10^9$  CFU/mL). Figures 1A and 1B represent the bacterial counts obtained from inoculum sizes of  $10^5$ ,  $10^7$  and  $10^9$  CFU/mL exposed to marbofloxacin with  $T_{MSW}$  of 0 and 25% respectively. The bacterial counts for experiments carried out with a  $T_{MSW}$  of 100% are shown in Fig. 1C or Fig. 1D depending on the susceptibility of surviving bacteria at the end of experiments. Whatever the initial inoculum size, all marbofloxacin regimens showed bactericidal activity during the first hours of exposure. Killing rates then declined with time until regrowth occurred, whatever the  $T_{MSW}$  and inoculum size. The minimal counts of surviving bacteria in the central flask seemed to increase with inoculum size, although the limit of detection of 100 CFU/mL prevented comparison of the  $10^5$  and  $10^7$  CFU/mL inocula (Table 1). Bacteria counts after 32 hours ranged from  $10^4$  to  $2.10^6$  CFU/mL when most of the surviving bacteria were susceptible to 0.128  $\mu\text{g/mL}$ , i.e. when they were not first-step mutants and ranged from  $5.10^7$  to  $6.10^8$  CFU/mL when most of the surviving bacteria were resistant to this concentration, i.e. when they have the same phenotype as first-step mutants. The higher regrowth associated with resistant bacteria selection may be explained by a higher growth rate or a slower rate of killing of resistant bacteria in the presence of marbofloxacin. A previously described integrated parameter, called ABBC (3), was used to assess marbofloxacin antimicrobial effect during the initial hours of exposure. It describes the ratio of areas from 0 to 10 hours delimited by time-kill curves in the absence and the presence of marbofloxacin

with the same inoculum sizes. Inoculum size had no net effect on ABBC (Table 1). However, slightly lower ABBC values and higher minimal counts were obtained with  $10^9$  CFU/mL inocula exposed to  $T_{MSW}$  of 100%, i.e. when resistant bacteria to 0.128  $\mu$ g/mL emerged compared to all other inoculum size/ $T_{MSW}$  combinations, suggesting that ABBC decrease might be related to resistant mutants selection. The detection limit might explain that no relation between ABBC and resistance selection was observed for  $10^5$  and  $10^7$  CFU/mL inocula. The relatively weak effect of inoculum size on fluoroquinolone antimicrobial activity observed in the present study is in agreement with a previous report on *Escherichia coli* exposure to ciprofloxacin or trovafloxacin in an *in vitro* pharmacodynamic model (4).

Bacteria growing in the presence of 0.016 (2xMIC), 0.128 (one dilution before MPC) and 0.256 (MPC)  $\mu$ g/mL marbofloxacin were counted before and 32 hours after exposure to marbofloxacin. The frequencies of resistant bacteria were determined by the ratio of bacteria counts growing in the presence and the absence of marbofloxacin. Before exposure to marbofloxacin, very few bacteria were resistant to 0.128  $\mu$ g/mL and resistance to 0.256  $\mu$ g/mL was detected in only one initial inoculum of  $10^9$  CFU/mL (Fig. 2). At the end of control experiments without antibiotic, no mutant resistant to 0.128  $\mu$ g/mL marbofloxacin was observed whatever the inoculum size. As shown in Fig. 2, bacteria exposed to  $T_{MSW}$  of 100% became mostly resistant to 0.128  $\mu$ g/mL in five experiments among nine. Most of these resistant bacteria were still susceptible to the MPC of marbofloxacin for *Escherichia coli* ATCC 25922 (0.256  $\mu$ g/mL) suggesting that these resistant populations corresponded to first-step mutants. The detection of first-step mutants when concentrations were maintained within the MSW is in agreement with previous studies (5, 11). However, resistant mutants emerged systematically in the three experiments carried out with  $10^9$  CFU/mL, but only in one among three for  $10^5$  and  $10^7$  CFU/mL inocula. . We calculated AUC/MPC ratios by dividing the AUC from 0 to 24 hours by the MPC. The observed AUC/MPC values associated with

94 prevention of mutant selection irrespective of inoculum size were 44-54 hours. A value of 22  
95 hours was previously reported as sufficient to prevent the emergence of mutants resistant to  
96 ciprofloxacin in large inocula ( $10^{10}$  CFU) of susceptible *Escherichia coli* strains (9).  
97 However, in two thirds of our experiments with inoculum sizes of  $10^5$  and  $10^7$  CFU/mL, an  
98 AUC/MPC of 9-12 hours was sufficient to prevent the emergence of resistant mutants. These  
99 results support the hypothesis that breakpoint values of PK/PD parameters associated with the  
100 MPC and MSW concepts for preventing the emergence of resistant mutants may depend on  
101 the size of exposed bacterial population present at the infection site.

102         In summary, our results confirmed that maintaining concentrations above the MPC  
103 prevents the emergence of resistance. However, the process of mutant selection within the  
104 MSW was not evenly linked to underexposure to antibiotics but also influenced by the  
105 presence of mutant before antibiotic treatment which is directly linked to the bacterial  
106 population size. The *in vivo* relevance of these *in vitro* results merits investigation in animal  
107 models of infection.

108  
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## LEGENDS

### Figure 1:

Observed viable counts of *Escherichia coli* ATCC 25922 following exposures of initial inoculum sizes of  $10^5$  (●),  $10^7$  (□) or  $10^9$  (▲) CFU/mL to concentrations of marbofloxacin inside the mutant selection window for 0 % (A), 25% (B) or 100% (C-D) of the time. For  $T_{MSW}$  of 100%, experiments in which surviving bacteria were mainly susceptible to 0.128 µg/mL are represented in (C) and those in which surviving bacteria were mainly resistant to 0.128 µg/mL are represented in (D). In A, B and C, each symbol represents the mean of 2 experiments. In D, symbols (●) and (□) represent results of one experiment and symbols (▲) represent the means of 3 experiments. Error bars show standard deviation. Dotted line indicates the lower limit of detection ( $2 \log_{10}$  CFU/mL) used for bacterial quantification.

### Figure 2:

Frequencies of bacteria resistant to 0.016 µg/ml (white bars), 0.128 µg/mL (dotted bars) and 0.256 µg/mL (black bars) before and after exposure of initial inoculum sizes of  $10^5$ ,  $10^7$  or  $10^9$  CFU/mL to control (one experiment per inoculum size) or to marbofloxacin concentrations within the mutant selection window for 100% (3 experiments per inoculum size), 25% (2 experiments per inoculum size) and 0 % (2 experiments per inoculum size) of the time. ○, ⊗, and ■ indicate that no bacteria resistant to 0.016, 0.128 or 0.256 µg/mL respectively were detected.

133 TABLE 1. Resistance selection and bactericidal activity of marbofloxacin.

Time in the MSW (%)	100					25			0		
Inoculum size (CFU/mL)	10 <sup>5</sup>		10 <sup>7</sup>		10 <sup>9</sup>	10 <sup>5</sup>	10 <sup>7</sup>	10 <sup>9</sup>	10 <sup>5</sup>	10 <sup>7</sup>	10 <sup>9</sup>
Susceptibility <sup>a</sup>	-	+	-	+	+	-	-	-	-	-	-
Minimal counts (CFU/mL)	<100	<100	<100	<100	2.10 <sup>4</sup>	<100	150	10 <sup>3</sup>	<100	<100	600
Final counts (CFU/mL)	2.10 <sup>6</sup>	1.10 <sup>8</sup>	7.10 <sup>4</sup>	6.10 <sup>8</sup>	5.10 <sup>7</sup>	6.10 <sup>4</sup>	8.10 <sup>4</sup>	3.10 <sup>4</sup>	7.10 <sup>3</sup>	2.10 <sup>4</sup>	1.10 <sup>4</sup>
ABBC (log CFU/mL.h)	53	51	60	56	44	54	56	53	54	57	51

134 <sup>a</sup> Susceptibility is assessed at the end of the experiments. Experiments in which the  
135 population is mainly resistant to 0.128 µg/mL are noted +, those mainly susceptible to 0.128  
136 µg/mL are noted -.

137      TABLE 2. Marbofloxacin pharmacokinetic parameters in relation to MIC, MPC and MSW.

Targeted time inside the MSW (%)	T <sub>&gt;MIC</sub> (%)	T <sub>&gt;MPC</sub> (%)	AUC/MPC
100	100	0	9-12
25	100	75	44-54
0	100	100	176-210

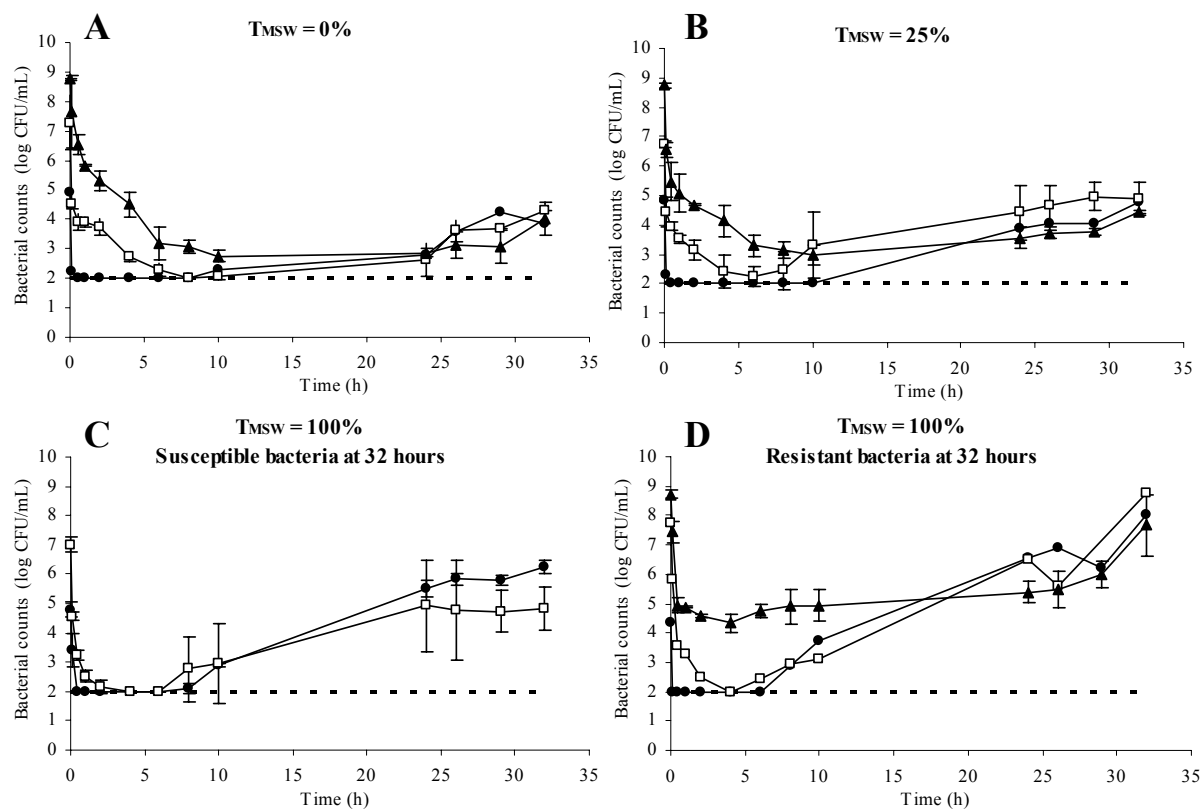
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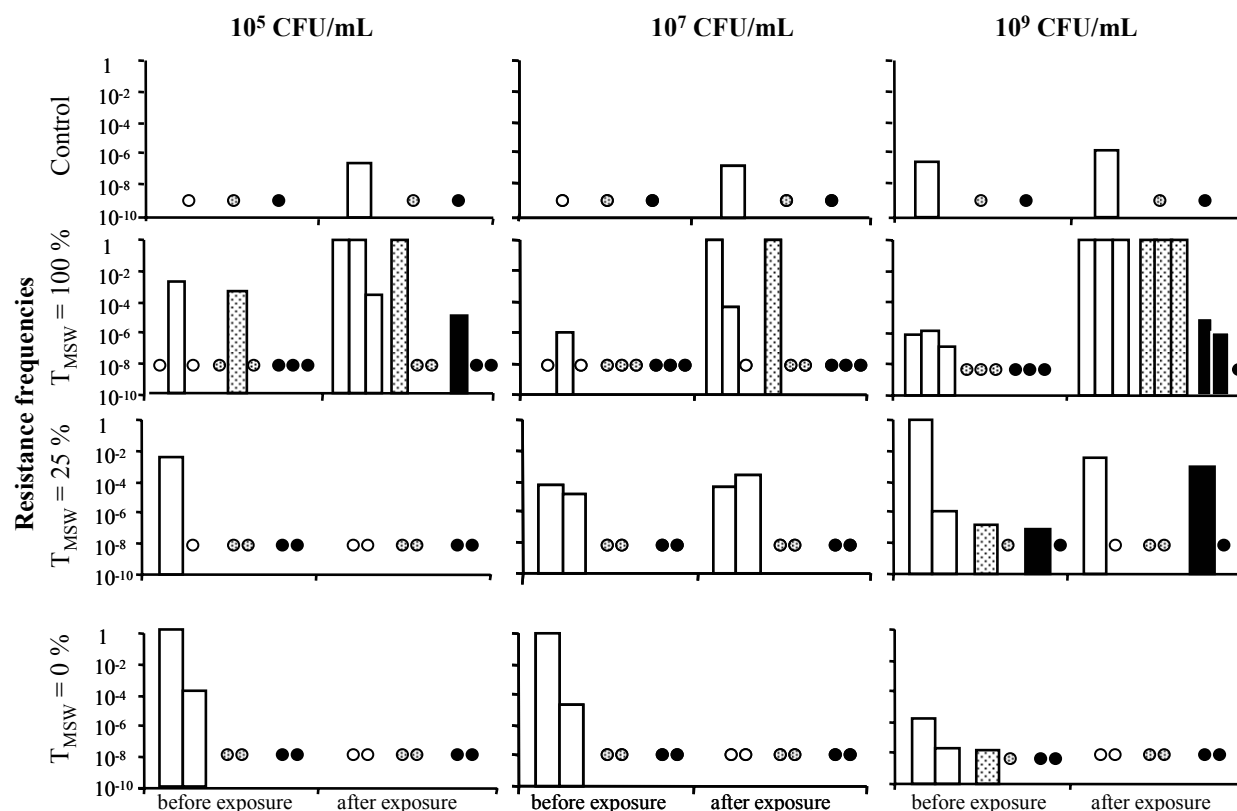
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142 **FIGURE 4**



143

144 FIGURE 5



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