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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

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

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

36 The aim of this study was to determine, using marbofloxacin a fluoroquinolone
37 extensively used in veterinary medicine, an interaction between inoculum sizes (10^5 , 10^7 or
38 10^9 CFU/mL) and marbofloxacin exposures characterized by different T_{MSW} (0, 25 or 100%)
39 on the selection of *Escherichia coli* resistant mutants.

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41 Marbofloxacin MIC for *Escherichia coli* ATCC 25922 was determined by
42 microdilution technique and MPC by a previously described method (1). MIC and MPC were
43 0.008 and 0.256 $\mu\text{g/mL}$, respectively.

44 Bacteria suspended in Mueller-Hinton (MH) broth were exposed in an *in vitro*
 45 pharmacodynamic model to three monoexponential kinetic profiles of marbofloxacin to
 46 ensure T_{MSW} of 0, 25 and 100% corresponding to times above MPC ($T_{>MPC}$) of 100, 75 and
 47 0%. Bacteria exposures to marbofloxacin were determined from serial samples by HPLC and
 48 killing and regrowth of bacterial population were assessed by counting viable bacteria.

49 For all initial inoculum sizes, bacterial counts without antibiotic revealed similar
 50 exponential growth rate until the carrying capacity of the *in vitro* system was reached (about
 51 10^9 CFU/mL). Figures 1A and 1B represent the bacterial counts obtained from inoculum sizes
 52 of 10^5 , 10^7 and 10^9 CFU/mL exposed to marbofloxacin with T_{MSW} of 0 and 25% respectively.
 53 The bacterial counts for experiments carried out with a T_{MSW} of 100% are shown in Fig. 1C or
 54 Fig. 1D depending on the susceptibility of surviving bacteria at the end of experiments.
 55 Whatever the initial inoculum size, all marbofloxacin regimens showed bactericidal activity
 56 during the first hours of exposure. Killing rates then declined with time until regrowth
 57 occurred, whatever the T_{MSW} and inoculum size. The minimal counts of surviving bacteria in
 58 the central flask seemed to increase with inoculum size, although the limit of detection of 100
 59 CFU/mL prevented comparison of the 10^5 and 10^7 CFU/mL inocula (Table 1). Bacteria
 60 counts after 32 hours ranged from 10^4 to 2.10^6 CFU/mL when most of the surviving bacteria
 61 were susceptible to 0.128 $\mu\text{g/mL}$, i.e. when they were not first-step mutants and ranged from
 62 5.10^7 to 6.10^8 CFU/mL when most of the surviving bacteria were resistant to this
 63 concentration, i.e. when they have the same phenotype as first-step mutants. The higher
 64 regrowth associated with resistant bacteria selection may be explained by a higher growth rate
 65 or a slower rate of killing of resistant bacteria in the presence of marbofloxacin. A previously
 66 described integrated parameter, called ABBC (3), was used to assess marbofloxacin
 67 antimicrobial effect during the initial hours of exposure. It describes the ratio of areas from 0
 68 to 10 hours delimited by time-kill curves in the absence and the presence of marbofloxacin

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

78 Bacteria growing in the presence of 0.016 (2xMIC), 0.128 (one dilution before MPC)
 79 and 0.256 (MPC) $\mu\text{g/mL}$ marbofloxacin were counted before and 32 hours after exposure to
 80 marbofloxacin. The frequencies of resistant bacteria were determined by the ratio of bacteria
 81 counts growing in the presence and the absence of marbofloxacin. Before exposure to
 82 marbofloxacin, very few bacteria were resistant to 0.128 $\mu\text{g/mL}$ and resistance to 0.256
 83 $\mu\text{g/mL}$ was detected in only one initial inoculum of 10^9 CFU/mL (Fig. 2). At the end of
 84 control experiments without antibiotic, no mutant resistant to 0.128 $\mu\text{g/mL}$ marbofloxacin
 85 was observed whatever the inoculum size. As shown in Fig. 2, bacteria exposed to T_{MSW} of
 86 100% became mostly resistant to 0.128 $\mu\text{g/mL}$ in five experiments among nine. Most of these
 87 resistant bacteria were still susceptible to the MPC of marbofloxacin for *Escherichia coli*
 88 ATCC 25922 (0.256 $\mu\text{g/mL}$) suggesting that these resistant populations corresponded to first-
 89 step mutants. The detection of first-step mutants when concentrations were maintained within
 90 the MSW is in agreement with previous studies (5, 11). However, resistant mutants emerged
 91 systematically in the three experiments carried out with 10^9 CFU/mL, but only in one among
 92 three for 10^5 and 10^7 CFU/mL inocula. . We calculated AUC/MPC ratios by dividing the
 93 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.

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113 **LEGENDS**

114 **Figure 1:**

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

125 **Figure 2:**

126 Frequencies of bacteria resistant to 0.016 $\mu\text{g/ml}$ (white bars), 0.128 $\mu\text{g/mL}$ (dotted
 127 bars) and 0.256 $\mu\text{g/mL}$ (black bars) before and after exposure of initial inoculum sizes of 10^5 ,
 128 10^7 or 10^9 CFU/mL to control (one experiment per inoculum size) or to marbofloxacin
 129 concentrations within the mutant selection window for 100% (3 experiments per inoculum
 130 size), 25% (2 experiments per inoculum size) and 0 % (2 experiments per inoculum size) of
 131 the time. ○, ⊗, and ■ indicate that no bacteria resistant to 0.016, 0.128 or 0.256 $\mu\text{g/mL}$
 132 respectively were detected.

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

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

134 ^a 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 _{>MIC} (%) | T _{>MPC} (%) | AUC/MPC |
|----------------------------------|--------------------------|--------------------------|---------|
| 100 | 100 | 0 | 9-12 |
| 25 | 100 | 75 | 44-54 |
| 0 | 100 | 100 | 176-210 |

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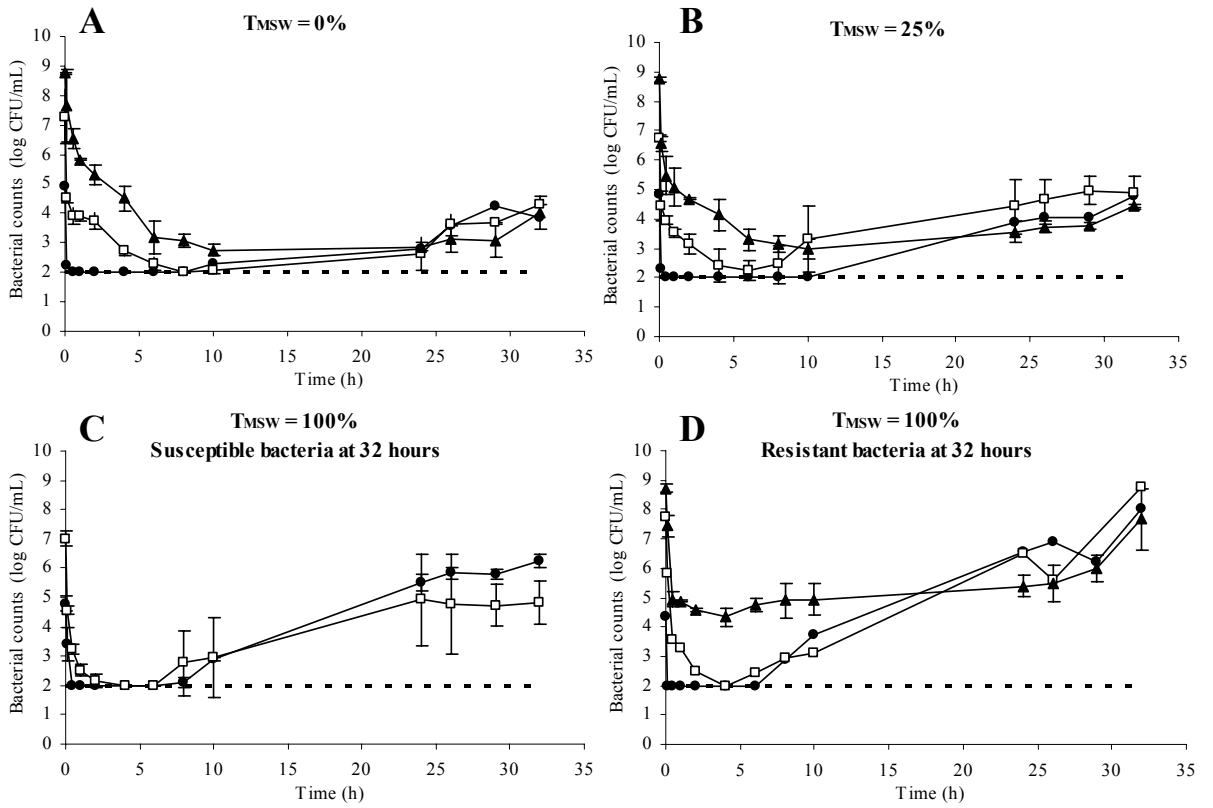
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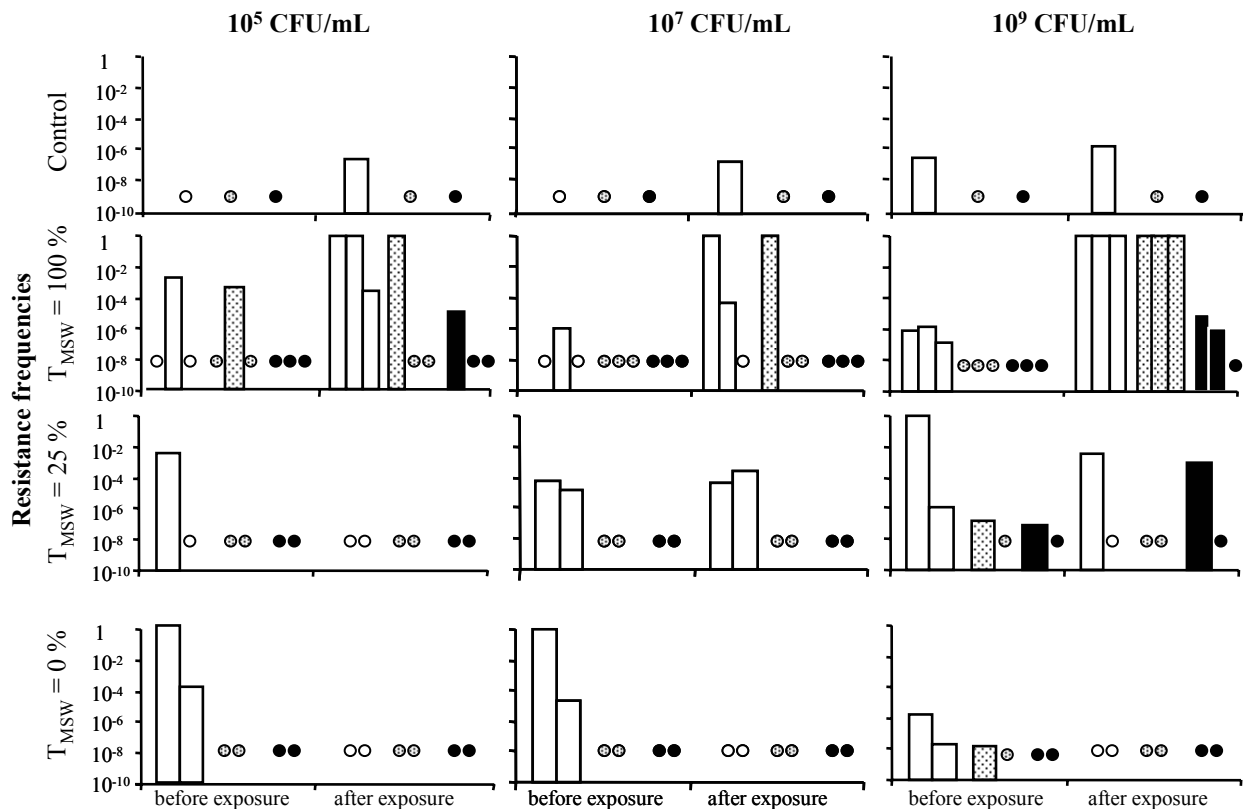
FIGURE 4



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FIGURE 5



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