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# Using *In Vitro* Dynamic Models To Evaluate Fluoroquinolone Activity against Emergence of Resistant *Salmonella enterica* Serovar Typhimurium

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**ABSTRACT** The objectives of this study were to determine pharmacokinetic/pharmacodynamic (PK/PD) indices of fluoroquinolones that minimize the emergence of resistant *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) using *in vitro* dynamic models and to establish mechanisms of resistance. Three fluoroquinolones, difloxacin (DIF), enrofloxacin (ENR), and marbofloxacin (MAR), at five dose levels and 3 days of treatment were simulated. Bacterial killing-regrowth kinetics and emergence of resistant bacteria after antibacterial drug exposure were quantified. PK/PD indices associated with different levels of antibacterial activity were computed. Mechanisms of fluoroquinolone resistance were determined by analyzing target mutations in the quinolone resistance-determining regions (QRDRs) and by analyzing overexpression of efflux pumps. Maximum losses in susceptibility of fluoroquinolone-exposed *S.* Typhimurium occurred at a simulated AUC/MIC ratio (area under the concentration-time curve over 24 h in the steady state divided by the MIC) of 47 to 71. Target mutations in *gyrA* (S83F) and overexpression of *acrAB-tolC* contributed to decreased susceptibility in fluoroquinolone-exposed *S.* Typhimurium. The current data suggest AUC/MIC (AUC/mutant prevention concentration [MPC])-dependent selection of resistant mutants of *S.* Typhimurium, with AUC/MPC ratios of 69 (DIF), 62 (ENR), and 39 (MAR) being protective against selection of resistant mutants. These values could not be achieved in veterinary clinical areas under the current recommended therapeutic doses of the fluoroquinolones, suggesting the need to reassess the current dosing regimen to include both clinical efficacy and minimization of emergence of resistant bacteria.

**KEYWORDS** efflux pumps, fluoroquinolone, global regulator, *in vitro* dynamic model, mutant prevention concentration, PK/PD integration, *Salmonella* Typhimurium, target mutation, *acrAB-tolC*, *gyrA*

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Fluoroquinolones are synthetic antibacterial agents with broad-spectrum, concentration-dependent bactericidal activity against a wide range of pathogens (1). However, resistance of human and animal pathogenic bacteria to a wide spectrum of antibacterial agents, including fluoroquinolones, is an increasing concern globally (2, 3). Furthermore, overuse or inappropriate use of antibiotics in animals, especially food-producing animals, can lead to transfer of bacterial resistance to humans via the food chain (4).

A common method for assessing the efficacy of antibacterial drugs focuses on evaluation of the effect of selected doses of the drug on the reduction in the total bacterial number at the infection site, with less attention to the amplification of

**TABLE 1** Antibacterial activity of three fluoroquinolones against *S.* Typhimurium isolates from pigs

| Parameter and isolate type | Value (μg/ml) for: | | |
|---|---|---|---|
| | DIF | ENR | MAR |
| Clinical isolates (*n*) | 16 | 16 | 16 |
| MIC (μg/ml) | | | |
| Range | 0.13–8 | 0.03–2 | 0.03–2 |
| $MIC_{50}$ | 1 | 0.25 | 0.13 |
| $MIC_{90}$ | 4 | 2 | 1 |
| R[a] (%) | 31.25 | 12.50 | 6.25 |
| MPC (μg/ml) | | | |
| Range | 0.50–16 | 0.13–4 | 0.13–4 |
| $MPC_{50}$ | 2 | 0.50 | 0.50 |
| $MPC_{90}$ | 8 | 4 | 4 |
| MPC/MIC | | | |
| Range | 4–8 | 4–8 | 4 |
| $(MPC/MIC)_{50}$ | 4 | 4 | 4 |
| $(MPC/MIC)_{90}$ | 8 | 8 | 4 |
| ATCC 14028[b] | | | |
| MIC | 0.13 | 0.03 | 0.03 |
| MPC | 0.50 | 0.13 | 0.13 |
| MIC/MPC | 4 | 4 | 4 |

[a]*R* (%), percent resistance in clinical isolates using the breakpoint of ciprofloxacin from the CLSI (M100-S23).
[b]The control strain used in the present study.

drug-resistant subpopulations of bacteria by selective pressure (5, 6). However, a number of studies have accentuated the importance of a mutant prevention concentration (MPC)-based dosing approaches to enhance the potency of antibiotics and restrict the selection of resistant mutants (7, 8). Furthermore, *in vitro* dynamic models that mimic antibacterial pharmacokinetics *in vivo* have been applied to bridge the gap between static *in vitro* data and the dynamic time course profiles. These models have been used to establish the relationship between fluoroquinolone exposure and emergence of resistant bacteria in humans, as well as to compare the potential of different drugs within the same class for restricting the selection of resistant mutants of bacteria (7, 9).

The aim of this study was to apply the MPC and *in vitro* dynamic model-based approaches to determine pharmacokinetic/pharmacodynamic (PK/PD) indices of fluoroquinolones that minimize the emergence of resistant *Salmonella enterica* serovar Typhimurium. We chose to investigate *S.* Typhimurium because of its zoonotic significance and the high level of drug resistance reported for this organism across the globe (10–12). Loss of susceptibility in laboratory and field *S.* Typhimurium isolates was associated with inappropriate use of fluoroquinolones (12). We investigated veterinary fluoroquinolones, including difloxacin (DIF), enrofloxacin (ENR), and marbofloxacin (MAR), for two reasons: first, the risk of cross-resistance for fluoroquinolones has been reported for both human and animal pathogens (3, 4, 13–15), and second, previous studies have demonstrated differences among veterinary fluoroquinolones in their activity and potential for selection of resistant mutants of bacteria (16). We further investigated the major mechanisms of fluoroquinolone resistance, including target mutations as well as the expression of the efflux pumps and global regulators, and established the relationships between these mechanisms and reduction in fluoroquinolone susceptibility.

(Part of this study was presented at the 13th International Congress of the European Association for Veterinary Pharmacology and Toxicology, 19 to 22 July 2015 [17]).

## RESULTS

**Antibacterial activity.** The pharmacodynamic profile of FQs against *S.* Typhimurium clinical isolates from pigs and a standard strain (ATCC 14028) were determined *in vitro*. As shown in Table 1, ENR and MAR had comparable activity, which was higher than that

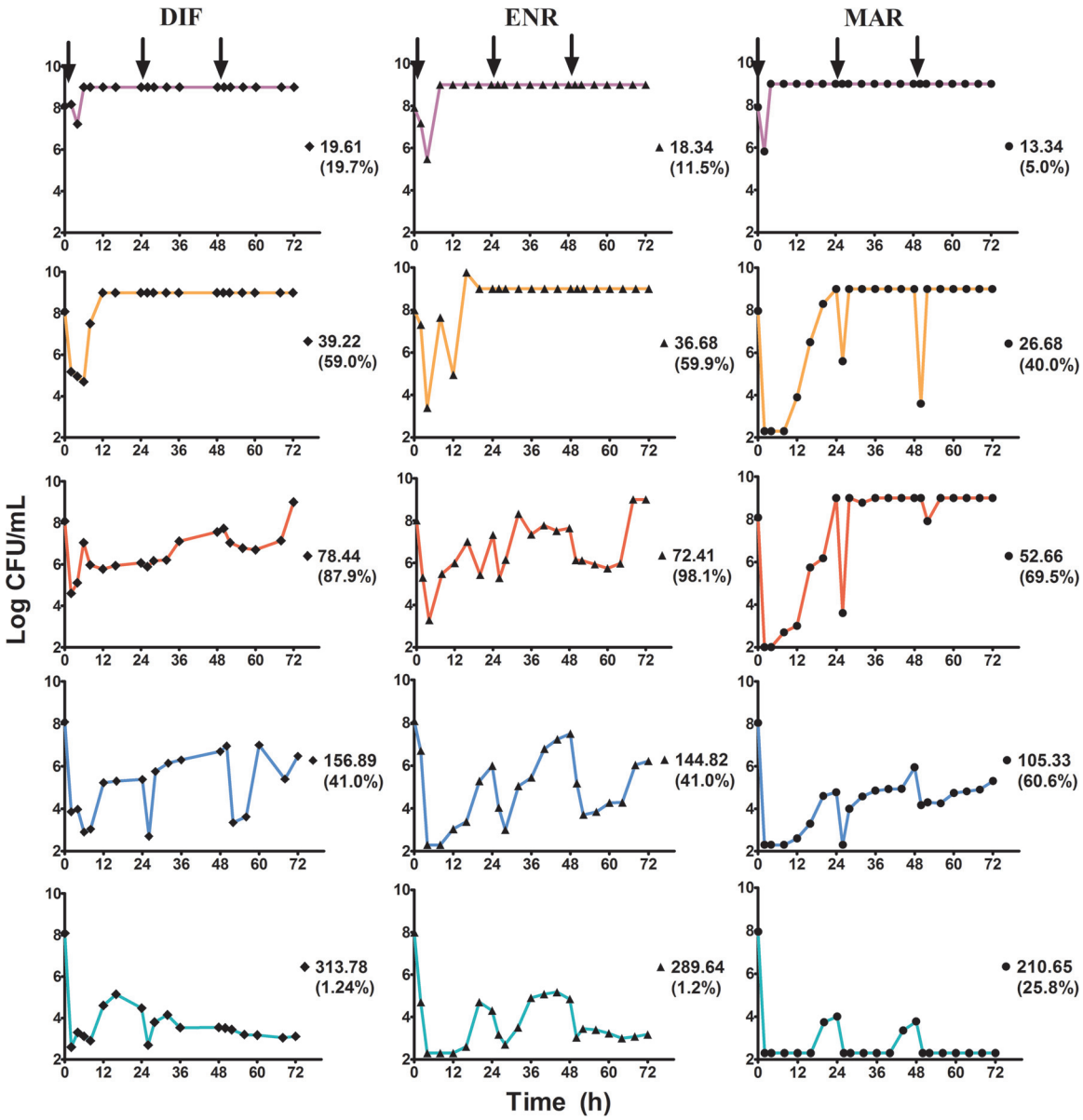


**FIG 1** Time courses of killing and regrowth kinetics for *S.* Typhimurium strain exposed to a 72-h course of difloxacin (DIF), enrofloxacin (ENR), and marbofloxacin (MAR). Values at the right side of each panel indicate the simulated AUC/MIC ratios and $T_{MSW}$. Arrows indicate fluoroquinolone dosing.

of DIF. $MPC_{50}$ and $MPC_{90}$ concentrations were 4-fold (DIF) and 2-fold (ENR and MAR) higher than the respective $MIC_{50}$ and $MIC_{90}$ values. These data suggested that ENR and MAR have a slightly higher potency than DIF.

**Kill-regrowth analysis.** The time course of killing and regrowth of *S.* Typhimurium incubated in the presence of one of the FQs at five different constantly changing concentrations was evaluated (Fig. 1). The antimicrobial activities of DIF, ENR, and MAR were evaluated at a range of AUC/MIC ratios (areas under the concentration-time curve over 24 h in the steady state divided by the MIC), including those achievable at the clinically recommended doses of all drugs, against *S.* Typhimurium. An increase in AUC/MIC ratio resulted in a decrease in the numbers of surviving pathogens. The intensity of the antimicrobial effect ($I_E$) versus AUC/MIC plots shows differences among the 3 FQs (Fig. 2). For example, at an AUC/MIC ratio of 125, which is the proposed AUC/MIC target for predicting the activity of FQs (18), the $I_E$ of MAR was 55% and 29%

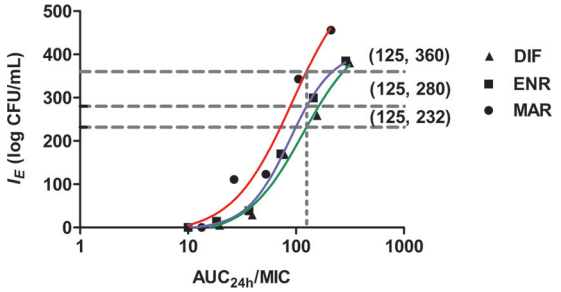


**FIG 2** AUC/MIC-dependent antibacterial effect of difloxacin (DIF), enrofloxacin (ENR), and marbofloxacin (MAR) against *S.* Typhimurium, fitted by equation 1. For DIF, $Y_{max}$ = 386.3, $dx$ = 2.617, and $x_0$ = 0.5054 ($R^2$ = 0.99); for ENR, $Y_{max}$ = 382.0, $dx$ = 2.462, and $x_0$ = 0.4494 ($R^2$ = 1); for MAR, $Y_{max}$ = 481.8, $dx$ = 2.486, and $x_0$ = 0.5196 ($R^2$ = 0.97).

greater than those of DIF and ENR, respectively, suggesting the greatest antimicrobial effect of a given AUC/MIC ratio of MAR against *S.* Typhimurium.

**Emergence of resistance.** The MIC values measured after each dosing (at 24, 48, and 72 h) were normalized to the initial (0 h) MIC, and the relationship between MIC changes and simulated AUC/MIC or AUC/MPC ratios was analyzed by using a mathematical equation. As shown in Fig. 3A, the maximum loss in susceptibility (about 8-fold

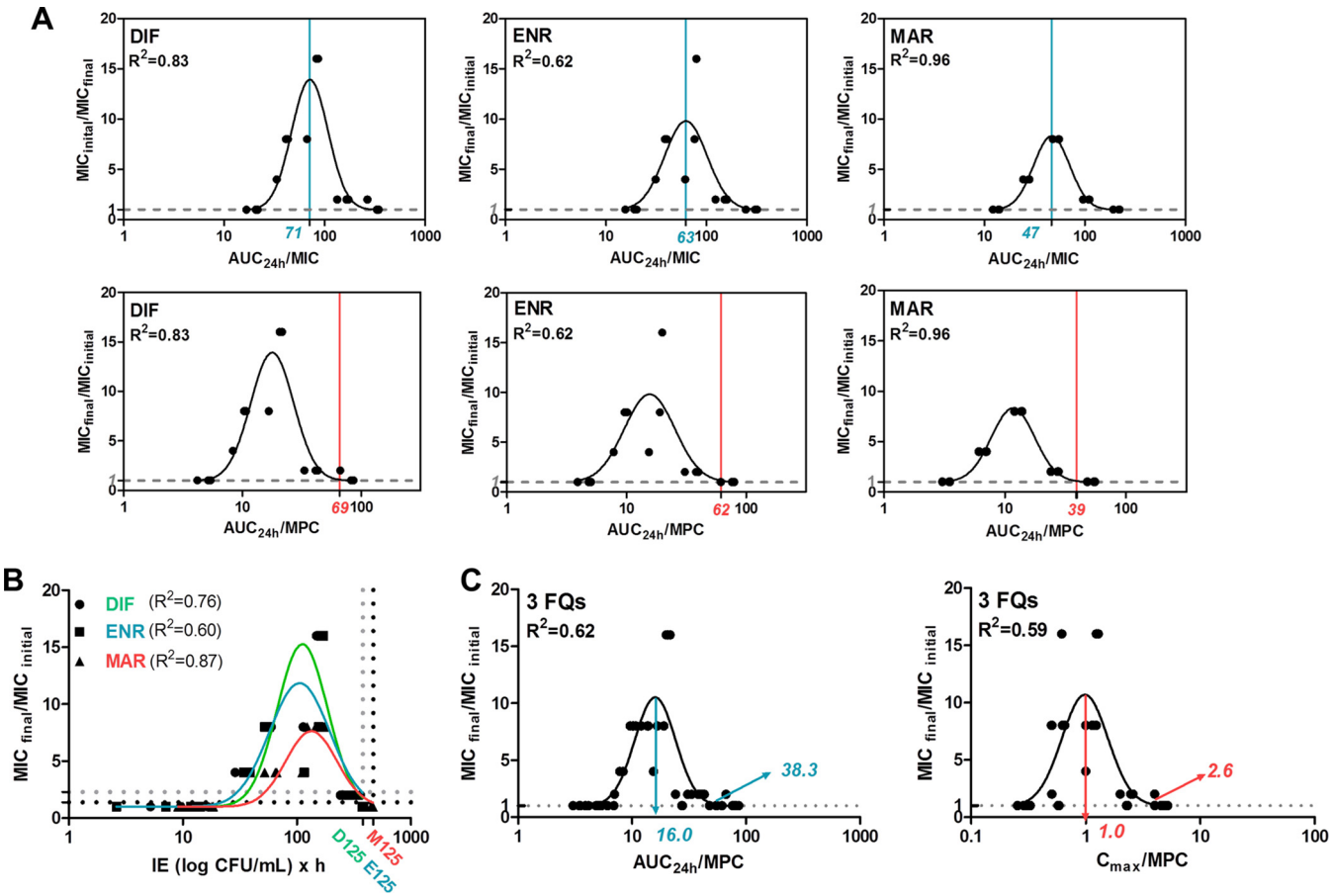


**FIG 3** Changes in susceptibility of *S.* Typhimurium. (A) Effect of $AUC_{24}$/MIC ratio (top) or $AUC_{24}$/MPC ratio (bottom) on the susceptibility of *S.* Typhimurium strains exposed to difloxacin (DIF), enrofloxacin (ENR), and marbofloxacin (MAR). Data were fitted by equation 2. (Top) For DIF, $a$ = 12.92, $x_c$ = 1.85, and $b$ = 0.18; for ENR, $a$ = 8.82, $x_c$ = 1.8, and $b$ = 0.21; for MAR, $a$ = 7.32, $x_c$ = 1.67, and $b$ = 0.18. (Bottom) For DIF, $a$ = 12.92, $x_c$ = 1.25, and $b$ = 0.18; for ENR, $a$ = 8.82, $x_c$ = 1.19, and $b$ = 0.21; for MAR, $a$ = 7.32, $x_c$ = 1.07, and $b$ = 0.18. (B) Resistance of *S.* Typhimurium related to the intensity of the antibacterial effect ($I_E$). Data were fitted by equation 2. For DIF, $a$ = 14.27, $x_c$ = 2.05, $b$ = 0.0.22, and $R^2$ = 0.76; for ENR, $a$ = 10.85, $x_c$ = 2.03, $b$ = 0.26, and $R^2$ = 0.60; for MAR, $a$ = 6.63, $x_c$ = 2.13, $b$ = 0.22, and $R^2$ = 0.87. (C) Resistance of *S.* Typhimurium related to the simulated $AUC_{24}$/MPC ratio (left) or $C_{max}$/MPC ratio (right) of three fluoroquinolones (3FQs; combined data). Data were fitted by equation 2. (Left) $a$ = 9.52, $x_c$ = 1.20, and $b$ = 0.18; (right) $a$ = 9.81, $x_c$ = −0.02, and $b$ = 0.21.

MIC increase for MAR and 16-fold MIC increase for DIF and ENR), which is the central point in the curve, was observed at AUC/MIC ratios of 47 (MAR; $R^2 = 0.98$), 63 (ENR; $R^2 = 0.62$), and 71 (DIF; $R^2 = 0.83$). No loss in susceptibility was observed at the lowest (13, 18, and 20) and highest (211, 290, and 314) simulated AUC/MIC ratios for MAR, ENR, and DIF. The estimated AUC/MPC values that may protect the selection of resistant *S.* Typhimurium were 69 (DIF), 62 (ENR), and 39 (MAR). The $I_E$-versus-resistance relationship was also analyzed using equation 2. As shown in Fig. 3B, a bell-shaped function describes this relationship, with the highest MIC increases observed at an $I_E$ value of 112 for DIF (range, 96 to 132; $R^2 = 0.76$), 106 for ENR (range, 84 to 133; $R^2 = 0.60$), and 134 for MAR (range, 119 to 151; $R^2 = 0.87$).

Similar patterns of the AUC/MIC-dependent changes in the susceptibility of *S.* Typhimurium to DIF, ENR, and MAR could allow establishment of a generalized relationship between MIC increase and AUC/MIC ratios of FQs. Therefore, combined data for MIC changes over a 3-day course of FQ treatment with DIF, ENR, and MAR were integrated with combined AUC/MPC and $C_{max}$/MPC (the maximum concentration of drug in serum divided by the MPC) values. As shown in Fig. 3C, no losses in susceptibility were observed when the combined AUC/MPC ratio was >38.3 and the $C_{max}$/MPC ratio was >2.6. This AUC/MPC value was greater than the individual values for MAR (1.4-fold) but less than that for DIF (1.8-fold) or ENR (1.6-fold).

**Population dynamics of fluoroquinolone treatment.** To ensure the residual activity of the drugs during the experiment, bioassays were conducted using filtrates taken from the culture medium, and MIC values of filtrates against the parent strain were converted to real-time drug residues in continuous culture. As shown in Fig. 4A, the MIC estimates of the filtrates slightly decreased with time. However, these values were less (only by 1- to 3-fold) than the anticipated drug concentration at a rate equal to the flow, suggesting density-dependent decay does not fully explain the failure of the simulated FQ concentration to clear the bacteria in the continuous culture.

The number of biofilm-forming bacteria was estimated from washes of the culture vessels before and after sonicating the vessels to release bacteria adhering to the walls. As shown in Fig. 4B, both the total population and subpopulation of bacteria recovered from the walls of the culture vessels were lower in cultures with drug, especially at the highest AUC/MIC ratios (314, 290, and 211 h for DIF, ENR, and MAR) compared with control cultures, suggesting AUC/MIC-dependent inhibition of biofilm formation in *S.* Typhimurium.

In order to predict and replicate the population dynamics of the treated continuous culture, a previously described mathematical model was used with hypothetical strains after expanding the equation to include an emerging mutant subpopulation (19–21). Figure 5C shows the changes in populations of the hypothetical weakly biofilm-forming strain in the presence of density-dependent decay at the effective concentrations of the FQ based on an AUC/MIC ratio for ENR of 63, AUC/MPC ratio for ENR of 62, and AUC/MPC ratio for three FQs of 38. There was maximal loss in susceptibility (Fig. 4C, left), similar to the continuous culture experiment (Fig. 1), whereas the selection of resistance was low (Fig. 4C, middle and right). These simulations show FQ AUC/MIC (AUC/MPC)-dependent anti-*Salmonella* activity similar to our earlier results, and the activity against selection of resistant *Salmonella* between ENR and three FQs combined was comparable.

**Mechanisms of resistance.** Mechanisms of resistance in clinical isolates and *in vitro*-selected resistant mutants of *S.* Typhimurium were elucidated (Table 2). As the highest fold increases in MICs were observed after repeat dosing, selections of representative first-step mutants were made from cultures after the last dosing (72 h). Eight to 32 mutant strains were recovered from selective plates. Five colonies per plate from each dose level were selected for MIC determination. A representative colony showing the highest stable MIC increases by repeat MIC determinations then was selected per dose level for further phenotype and genotype assays. These isolates (designated STM-D1, -D2, -D3, and -D4) from cultures with AUC/MPC ratios of 9 to 72, isolates from

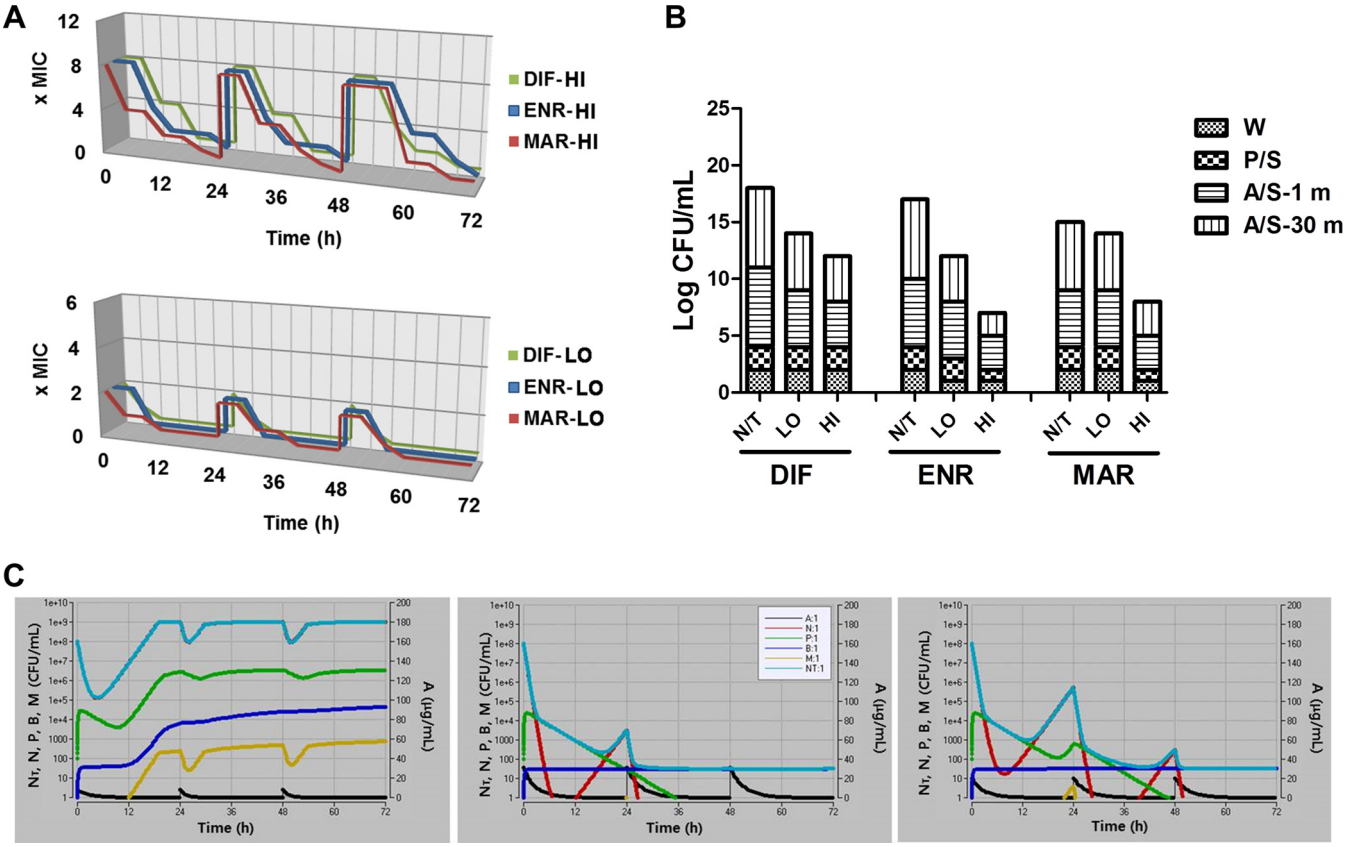


**FIG 4** Population dynamics of fluoroquinolone treatment. (A) Bioassay estimates of the MICs of DIF, ENR, and MAR during 3-day continuous culture. The culture medium was taken from the central vessel every 4 h. After removing the bacteria through filtration, MIC values were checked with the *S.* Typhimurium standard strain. LO, fluoroquinolone treatment with AUC/MIC ratio of 53 to 78; HI, fluoroquinolone treatment with AUC/MIC ratio of 211 to 314. (B) Biofilm formation of *S.* Typhimurium following daily dosing with difoxacin, enrofloxacin, or difloxacin. At the end of the experiment (72 h), the culture vessel compartments were separated, and biofilm strains in the vessel were washed and suspended with PBS or MHB II with or without sonication. Mean viable cell densities of bacteria released from the walls of the culture vessels were counted. N/T, no antibiotic treatment; LO, treatment at AUC/MIC ratio of 53 to 78; HI, treatment with at AUC/MIC ratio of 211 to 314; WO, the densities of bacteria in PBS following the third wash with PBS; P/S, the densities estimated in MHB II prior to sonication; A/S-1 m or 30 m, the densities estimated in MHB II with sonication for 1 or 30 min. Three independent estimates of densities were made for each sample. (C) Simulation of fluoroquinolone treatment with parameter A with 72 h of continuous culture of *S.* Typhimurium, showing changes in viable cell density with fluoroquinolone concentration. A dose of ENR (AUC/MIC of ENR, 63 [left]; AUC/MPC of ENR, 62 [middle]; AUC/MPC of three fluoroquinolones, 38 [right]) administered every 24 h starting with a cell density of $10^8$. Standard parameters and their definitions are as described in Levin and Udekwu (20), with the following additions and changes in parameters: initA = 8, 31, or 20 $\mu$g; initN = 1e8; initR = 1; initD = 0; init *P* = 0; initB = 0; initM (density of mutant population) = 0; psimax = 1; psimin = −2; psimaxp = 0.001; psiminp = −0.01; psimaxb = 0.001; psiminb = −0.00001; psimaxm (maximum growth rate, mutant population) = 1; psiminm = −3; Mmin = 1.875; Mmax = 1.875; pd = 0; cr = 0.9; k = 2.261; e = 5e−7; kr = 0.25; kmax = 1e7; w = 3.9; wb = 0.0001; wm (flow rate of mutant population) = w; C = 500; f = 0; yp = 0.0001; yd = 0.00001; yb = 0.000001; yw = 0.0000001; ym (rate at which mutations occur in NT) = 0.000000001; ddx = 0.01; dv = 1e−9; dk = 1e−9. A1, antibiotic decay; N1, density of viable planktonic sensitive bacteria; P1, density of planktonic persistent bacteria; B1, density of the wall (biofilm) population; M1, density of mutant population; NT, total viable planktonic bacteria. The simulation was run by the Berkeley Madonna program, accessed from http://www.eclf.net.

the MPC plates of MAR, as well as 6 to 31% of clinical isolates (Table 1) had increased MICs (2- to 64-fold increases) for one or more of the FQs. MIC increases were transferable between FQs. This is in accordance with our previous findings in which losses in susceptibility of *Escherichia coli* exposed to one FQ were transferable to other FQs and even to structurally unrelated drugs, partly due to activation of global drug efflux pumps (16, 22). To confirm the contribution of efflux pump activity to this resistance, MICs were assessed in the presence of the efflux pump inhibitor phenylalanine-arginine-$\beta$-naphthylamide (PA$\beta$N). The MICs of the three FQs decreased by 2- to 8-fold in the presence of PA$\beta$N, indicating the involvement of efflux pumps for the observed resistance. Therefore, overexpression of the efflux transporter gene and global regulators was further evaluated by quantitative reverse transcription-PCR (RT-PCR). As shown in Fig. 5A, expression of *acrAB-tolC* mRNA increased in isolates STM-D2 and -D3 exposed to FQ AUC/MPC ratios of 6.7 and 13.2 ($P < 0.05$), while no drug exposure-related differences in *acrAB-tolC* expression were observed in isolates STM-D1, -D4, and -D5

**TABLE 2** Fluoroquinolone resistance phenotypes and amino acid substitutions in *in vitro*-selected mutants and clinical isolates of *S*. Typhimurium

| Strain | Fluoroquinolone MIC ($\mu$g/ml)[a] | | | | | | Mutation | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | DIF | DIF$^{EPI}$ | ENR | ENR$^{EPI}$ | MAR | MAR$^{EPI}$ | *gyrA* | *gyrB* | *parC* | *parE* |
| Parental strain | | | | | | | | | | |
| STM-st | 0.13 | <0.03 | 0.03 | <0.03 | 0.03 | <0.03 | WT | WT | WT | WT |
| Single-step mutant from the dynamic model[b] | | | | | | | | | | |
| STM-D2 | 4 | 2 | 1.00 | 1 | 0.50 | 1 | S83F | WT | WT | WT |
| STM-D3 | 2 | 2 | 4 | 2 | 2 | 1 | S83F | WT | WT | WT |
| STM-D4 | 1.00 | 0.50 | 0.50 | 0.25 | 0.50 | 0.25 | S83F | WT | WT | WT |
| STM-D5 | 0.25 | 0.25 | 0.06 | 0.06 | 0.06 | 0.06 | WT | WT | WT | WT |
| Single-step mutant from MPC plates | | | | | | | | | | |
| STM-mu-p1 | 1 | 0.25 | 0.50 | 0.25 | 0.50 | 0.25 | S83F | WT | WT | WT |
| Resistant clinical strains | | | | | | | | | | |
| STM-isol-2 | 0.25 | 0.06 | 0.06 | 0.03 | 0.03 | <0.03 | WT | WT | WT | WT |
| STM-isol-11 | 0.50 | 0.25 | 0.25 | 0.06 | 0.25 | 0.06 | D87Y | WT | WT | WT |
| STM-isol-15 | 4 | 0.5 | 2 | 0.25 | 2 | 0.25 | D87Y | WT | WT | WT |

[a]The MIC values were checked in the presence of 40 $\mu$g/ml of Phe-Arg-$\beta$-naphthylamide (PA$\beta$N), which is an efflux pump inhibitor (EPI).

[b]Representative single-step mutants were selected randomly from the dynamic model with AUC/MPC ratios of 6.7 (STM-D2), 13.2 (STM-D3), 26.3 (STM-D4), and 52.7 (STM-D5) with MAR.

exposed to FQ AUC/MPC ratios of 3.3, 26.3, and 52.7, respectively. Overexpression of global regulatory genes was also significantly increased at 72 h in isolates from the dynamic model, with AUC/MPC ratios of 6.7 and 13.2 increasing by up to 2- to 3-fold (*marA*), 3- to 3.5-fold (*soxS*), 5- to 10-fold (*ramA*), and 6- to 7-fold (*rob*) compared to that of the original strain, suggesting a positive correlation between gene expression of *acrAB-tolC* and its regulators (Fig. 5B), and the expression of *acrAB-tolC* in the STM-D3 strain was similar to that in STM-isolate-15 ($P < 0.05$), showing resistance (Fig. 5C).

Mutation in the quinolone resistance-determining regions (QRDRs) was also examined. Fully quinolone-susceptible strains had no mutations in the target genes. A single *gyrA* mutation with S83F was present in *in vitro*-selected resistant isolates but not in resistant clinical isolates (D87Y). No mutations were found in *gyrB*, *parC*, and *parE* in any strains. Interestingly, *in vitro*-selected isolates showed multiple mechanisms of resistance, including mutation and efflux pump overexpression, while in clinically resistant isolates, resistance was mainly attributed to target mutation.

**Relationships of MPC with pharmacokinetics.** *In vitro* pharmacodynamic data from the present study (MICs and MPCs) and published pharmacokinetic information of the three FQs in pigs (AUC and $C_{max}$) were integrated to evaluate whether PK/PD indices by conventional dosing regimen of FQs agree with the protective AUC/MIC or AUC/MPC ratios obtained in the present study (Table 3).

Based on Clinical and Laboratory Standards Institute (CLSI) breakpoints of ciprofloxacin (23), the conventional dosing was shown to be effective for the susceptible, but not intermediate and resistant, *in vitro* isolates. On the other hand, AUC/MIC and $C_{max}$/MIC ratios were not effective for the clinical isolates. In order to estimate an MPC-based dosing regimen, AUC/MPC ratio, $C_{max}$/MPC ratio, and T > MPC (the time during which plasma concentrations exceed the mutant prevention concentration) surrogates were analyzed. Analysis was performed to determine whether the conventional doses of the three FQs could prevent the growth of the least susceptible *Salmonella* first-step mutants. Our results showed that the order of potency of three FQs against *S*. Typhimurium based on AUC/MPC ratios was MAR > ENR > DIF (Table 3). While only MAR at the highest doses could achieve high levels of AUC/MPC$_{50}$ ratios, $C_{max}$/MPC$_{50}$ ratios, and T > MPC$_{50}$, the AUC/MPC$_{90}$ ratios and T > MPC$_{90}$ of the three FQs were below the protective values obtained here.

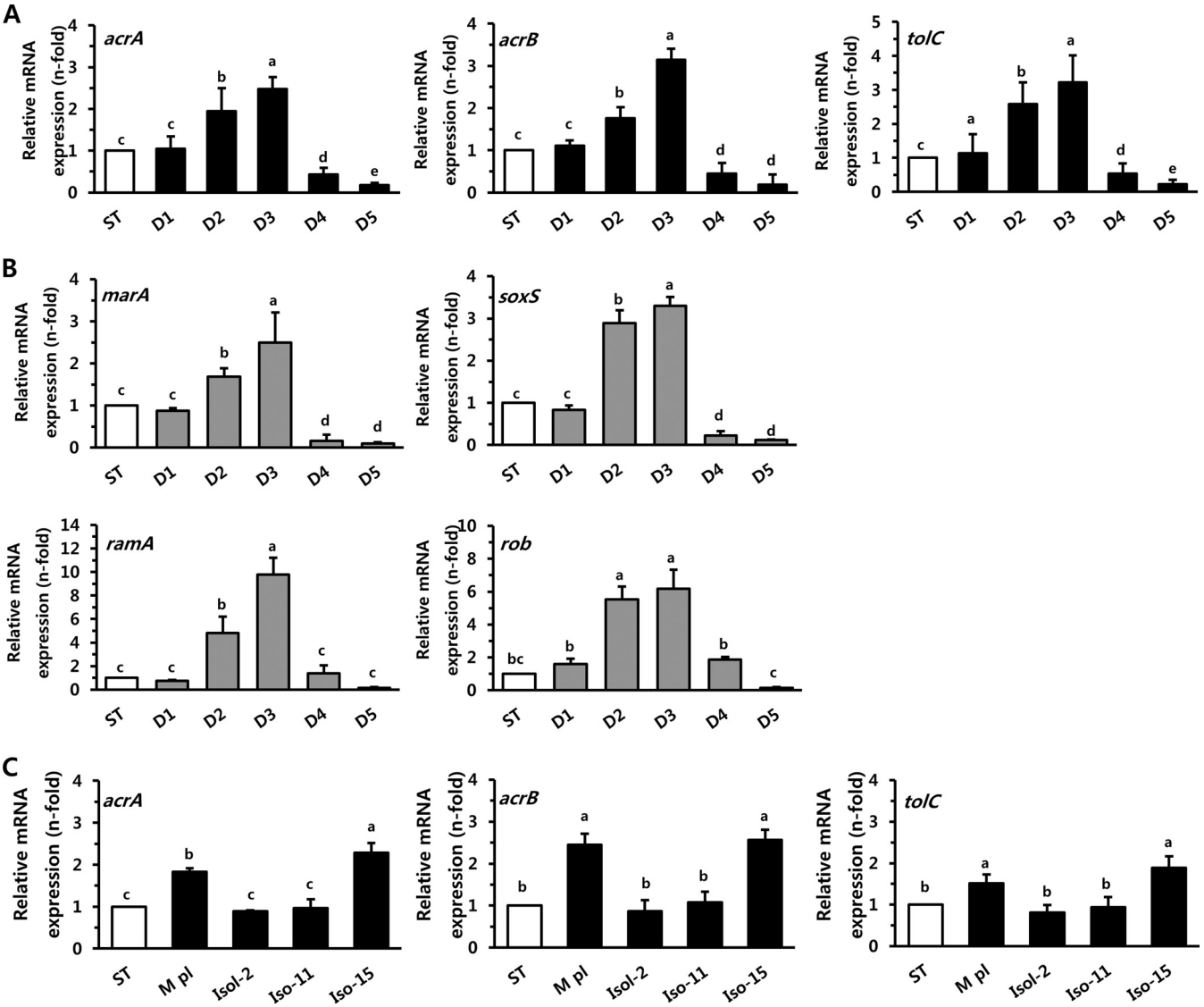


**FIG 5** mRNA expression levels of efflux pump (A) and regulator genes (B) for *in vitro* isolates and clinical isolates (C) of *S.* Typhimurium. Strains were randomly selected from the dynamic model treated with MAR at the end of the experiment (72 h). Strains D1 to D5 were isolated from cultures treated with AUC/MPC ratios of 3.3, 6.7, 13.2, 26.3, and 52.7, respectively. Data represent the means ± standard deviations from three independent experiments. Bars with different letters (a to e) show significant differences between groups ($P < 0.05$).

## DISCUSSION

The *in vitro* dynamic model approach was applied to measure the activity of three veterinary FQs against the emergence of resistance in *S.* Typhimurium. In the present study, *S.* Typhimurium ATCC 14028 was used with consideration of its susceptibility and its full genomic and phenotypic characterization.

The anti-*Salmonella* activities of the three FQs revealed an FQ-specific relationship between $I_E$ and AUC/MIC ratios. This was in agreement with our previous findings using FQs against *E. coli* and *Actinobacillus pleuropneumoniae* (16, 22, 24). Furthermore, the $I_E$ showed species specificity that was lower (8 to 41%) than those from reports using non-biofilm-forming bacteria, while it was similar to $I_E$s of FQs against biofilm-forming bacteria (difference of 8 to 10%) (16, 22, 24). The failure of FQs to suppress *Salmonella* growth in the culture could result not only from resistant populations but also from biofilm formation. However, the test strain used here was not a strong biofilm-forming strain, suggesting the role of AUC/MIC ratio-dependent losses in susceptibility upon continuous exposure to the FQs. The AUC/MPC value was suggested to be more

**TABLE 3** Pharmacodynamic predictors of fluoroquinolone activity based on integration of pharmacokinetic/pharmacodynamic indices against *S.* Typhimurium clinical isolates

| Parameters | Value for antibacterial agent | | | |
|---|---|---|---|---|
| | DIF | ENR | | MAR |
| | | Low dose | High dose | |
| Pharmacokinetic parameters[a] | | | | |
| Dose (mg/kg) | 5.00 | 2.50 | 10.00 | 2.00 |
| AUC ($\mu$g/ml) | 32.12 | 6.82 | 10.07 | 10.95 |
| $C_{max}$ ($\mu$g/ml) | 2.56 | 1.42 | 1.82 | 0.98 |
| $t_{max}$ (h) | 1.32 | 4.99 | 3.79 | 2.11 |
| $t_{1/2\beta}$ (h) | 18.09 | 6.56 | 10.68 | 7.80 |
| PK/PD integration based on MIC breakpoint[b] | | | | |
| $AUC/MIC_{BPs}$ | 513.92 | 109.12 | 161.12 | 175.20 |
| $AUC/MIC_{BPi}$ | 32.12 | 6.82 | 10.07 | 10.95 |
| $AUC/MIC_{BPr}$ | 16.06 | 3.41 | 5.04 | 5.48 |
| $C_{max}/MIC_{BPs}$ | 40.96 | 22.72 | 29.12 | 15.68 |
| $C_{max}/MIC_{BPi}$ | 2.56 | 1.42 | 1.82 | 0.98 |
| $C_{max}/MIC_{BPr}$ | 1.28 | 0.71 | 0.91 | 0.49 |
| PK/PD integration based on MIC of all clinical isolates[c] | | | | |
| $AUC/MIC_{50}$ | 32.12 | 27.28 | 40.28 | 84.23 |
| $AUC/MIC_{90}$ | 8.03 | 3.41 | 5.04 | 10.95 |
| $C_{max}/MIC_{50}$ | 2.56 | 5.68 | 7.28 | 7.54 |
| $C_{max}/MIC_{90}$ | 0.64 | 0.71 | 0.91 | 0.98 |
| PK/PD integration based on MPCs of susceptible isolates[d] | | | | |
| $AUC/MPC_{50}$ | 32.12 | 27.28 | 40.28 | 43.80 |
| $AUC/MPC_{90}$ | 16.06 | 13.64 | 20.14 | 21.90 |
| $C_{max}/MPC_{50}$ | 5.26 | 5.68 | 7.28 | 3.92 |
| $C_{max}/MPC_{90}$ | 1.28 | 2.84 | 3.64 | 1.96 |
| $T > MPC_{50}$ (h) | 24.59 | 20.36 | 38.37 | 15.37 |
| $T > MPC_{90}$ (h) | 6.46 | 11.53 | 23.97 | 7.57 |

[a]Dose, AUC, $C_{max}$, $t_{max}$, and $t_{1/2}$ were from published data (given in the text) from pharmacokinetic studies of each fluoroquinolone in healthy pigs at the clinical dose.
[b]The breakpoint of ciprofloxacin against all *Salmonella* species used was from the CLSI (M100-S23). BPs, breakpoint for susceptible ($\leq$0.06 $\mu$g/ml); BPi, breakpoint for intermediate (0.12 to 1.0 $\mu$g/ml); BPr, breakpoint for resistant ($\geq$2.0 $\mu$g/ml).
[c]MICs of all clinical isolates from the present study were used.
[d]MPCs of fluoroquinolone-susceptible clinical isolates (MIC of $\leq$0.06 $\mu$g/ml) from the present study were used.

accurate than the AUC/MIC value for the prevention of mutant selection, because the MPC concept includes a mutant subpopulation (7). In the present study, AUC/MPC ratios to reduce *Salmonella*-resistant mutants were 69 (DIF), 62 (ENR), and 39 (MAR). These results were consistent with previous reports (22, 24). However, in the current study, no difference of potency was found through conversion from AUC/MIC ratios to AUC/MPC ratios resulting from different MPC/MIC ratios, because the MPCs of all 3 FQs were 4 times higher than their respective MICs.

One interesting observation in this study was the differences in resistance mechanisms between *in vitro*-selected resistant mutants and resistant clinical isolates: mutation-dependent resistance (*in vitro* isolates) and efflux pump expression-dependent resistance (clinical isolates). Similarly, previous studies had also indicated that while field isolates or *in vivo*-selected mutants show small fold increases in MICs and often a single *gyrA* mutation, *S.* Typhimurium experimental mutants selected *in vitro* possess severalfold increases in MIC and multiple resistance mechanisms (25–27). In *Salmonella*, single *gyrA* mutations, in either S83F or D87Y, were associated with FQ-resistant isolates from humans and animals (10–12). Moreover, all field isolates harbored a single *gyrA* mutation of D87Y, which shows a lower MIC than mutation of S83F, while *in vitro*-selected resistance had an S83F mutation. Although no biochem-

istry was performed to confirm that this mutation conferred quinolone resistance, the mutation conferring a substitution at S83F is more abundant than at D87Y *in vitro*.

It has been suggested that the overexpression of efflux pumps is a sufficient condition for development of FQ resistance (28, 29). Furthermore, it has been reported that in the absence of *acrAB-tolC*, *S*. Typhimurium was unlikely to select mutation under ciprofloxacin pressure (30). In a resistant strain isolated from dynamic culture (STM-D3) and a clinical isolate (STM-isol-15), increases (2.5- to 3-fold) in *acrAB-tolC* mRNA expression and *gyrA* mutation were found, whereas the susceptible isolates showed suppressed *acrAB-tolC* gene expression and no *gyrA* mutation. These results indicate that a dosing strategy based on AUC/MPC (>39) was effective at preventing FQ resistance due to suppression of efflux pump overexpression and target mutation. The transcriptional activators, such as *marA*, *soxS*, *ramA*, and *rob*, have been reported to play a critical role in efflux pump activity in *Salmonella* (30–32). Consistent with this, there was a positive correlation in expression levels of *acrAB-tolC* and each regulator in isolates from an *in vitro* dynamic model in which downregulated efflux pump expression was associated with suppressed global regulators.

The AUC/MIC ratio of 125 to 250 and $C_{max}$/MIC ratio of greater than 8 to 10 were suggested to be the target PK/PD indices associated with efficacy of FQ treatment (18). Based on these endpoints, the three FQs could show high efficacy against susceptible clinical isolates, with AUC/MIC and $C_{max}$/MIC ratios of greater than 160 and 15, respectively. However, these ratios had to be much lower to show efficacy against strains with intermediate susceptibility. Moreover, we integrated our MPC data with the AUC and $C_{max}$ values achievable at the clinically recommended doses of these drugs in pigs (Table 3), and when these values were compared with the PK/PD predictions (Fig. 3) and the AUC/MPC ratio obtained from the present study, only MAR, with an AUC/$MPC_{50}$ ratio of 43.8, could achieve protection against the emergence of resistance in susceptible clinical isolates of *S*. Typhimurium. These findings suggest the applicability of the MPC-based dosing strategy for *S*. Typhimurium isolates only at higher doses of these agents and a better *in vitro* activity of a given AUC/MIC (AUC/MPC) ratio of MAR than those of DIF and ENR in terms of both antibacterial effect and prevention of the selection of mutants.

There are limitations to the use of MPC-based dosing, including the different drug concentration thresholds to restrict amplification of drug-resistant mutants depending on inoculum size and the chance for selection of a two-step mutation (8). Moreover, while MPC-based approaches can be useful to compare the abilities of FQs to prevent selection of *S*. Typhimurium mutants, the results of higher clinical dose requirements should be interpreted with caution, because inherently the *in vitro* MPC approach does not take into account the synergistic role of the immune system for clearing infections *in vivo* (7).

In summary, our results confirmed an AUC/MIC-dependent antibacterial activity and AUC/MPC-dependent selection of *S*. Typhimurium mutants in which maximum losses in FQ susceptibility occurred at simulated AUC/MIC ratios of 71 (DIF), 63 (ENR), and 47 (MAR). AUC/MPC ratios of 69 (DIF), 62 (ENR), and 39 (MAR) were estimated to be the minimum protective values against the selection of mutants. Generally, higher *in vitro* activity of a given AUC/MIC (AUC/MPC) ratio of MAR than DIF or ENR, in terms of both antibacterial effect and prevention of the selection of mutants, was observed. Both target mutations (*gyrA*; S83F) and overexpression of efflux pumps (*acrAB-tolC*) upregulated by global regulators contributed to these losses in susceptibility. Integration of the MIC and MPC data with published pharmacokinetic information in pigs revealed the inadequacy of the conventional dosing regimen of all three FQs in attaining the above-described protective values for 90% of the mutant subpopulation (AUC/$MPC_{90}$), suggesting the need to reassess the current dosing regimens of the FQs to include both clinical efficacy and minimization of emergence of resistant bacteria.

## MATERIALS AND METHODS

**Bacterial strains and antimicrobial agents.** A standard strain (ATCC 14028) and 16 clinical isolates of *S*. Typhimurium from diseased pigs were used. Three fluoroquinolones (FQs), including difloxacin (DIF),

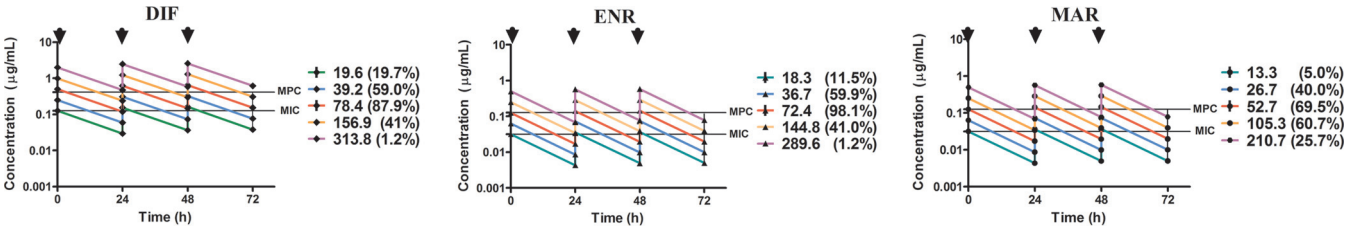


**FIG 6** *In vitro* simulated pharmacokinetic profiles of difloxacin (DIF), enrofloxacin (ENR), and marbofloxacin (MAR). Legends at the right indicate the AUC/MIC values and the percentage of the dosing interval in which fluoroquinolone concentrations fall within the mutant selection window ($T_{MSW}$). Daily doses of the fluoroquinolones for 3 consecutive days were simulated.

enrofloxacin (ENR), and marbofloxacin (MAR), were purchased from Sigma-Aldrich. Stock solutions (1 mg/ml in 0.1 N NaOH) were prepared and stored at −20°C. Working solutions were prepared daily from stock solution by appropriate dilutions with the culture medium.

**MIC and MPC determination.** Susceptibility tests were performed before, during, and after a 3-day course of treatment with the three FQs. The MICs were determined by the broth microdilution method according to the CLSI (23). MICs of a quality control organism, *Escherichia coli* (ATCC 25922), were also determined in parallel. MPCs were determined as described previously (9, 16). The MPC was defined as the lowest drug concentration that completely suppressed growth for 72 h. All experiments were performed in triplicate.

**Simulated pharmacokinetic profiles.** Single daily doses of the three FQs for 3 consecutive days with simulated half-lives ($t_{1/2}$) consistent with those reported in pigs were used: 12.5 h for DIF (33, 34), 10.6 h for ENR (35, 36) and 8 h for MAR (37). The doses were selected to include the achievable area under the curve (AUC) and maximum plasma concentration ($C_{max}$) over a 24-h dosing interval at the recommended clinical doses (1 to 5 mg/kg of body weight for DIF, 2.5 to 5 mg/kg for ENR, and 2 mg/kg for MAR) in pigs. The average ratios of the simulated AUC/MIC (averages of the values reached during 3 days) varied from approximately 13 to 20 and 210 to 314 for the three drugs (Fig. 6). These simulated AUC/MIC ratios corresponded to a $C_{max}$ that either equaled the MIC, fell between the MIC and MPC (i.e., within the mutant selection window, or MSW), or exceeded the MPC.

***In vitro* dynamic model.** A previously described dynamic model (9, 16, 22, 38, 39) was used in this study. In brief, the model consisted of three connected flasks: a dilution compartment containing fresh Mueller-Hinton broth II (MHB II) broth, a central compartment with either a bacterial culture alone (control growth experiment) or a bacterial culture in medium containing drug (killing-regrowth experiments), and an elimination compartment containing waste broth and bacteria. Peristaltic pumps (Masterflex; Cole-Parmer, USA) circulated in one direction, from the dilution compartment to the central compartment and from the central compartment to the elimination compartment, at a flow rate of 3.2 ml/h for DIF, 3.9 ml/h for ENR, and 5 ml/h for MAR. The temperature of the system was maintained at 37°C throughout the experiments. An overnight culture of *S.* Typhimurium was used to inoculate the central unit. After a 2-h incubation, the resulting exponentially growing bacterial cultures reached $10^7$ CFU/ml ($6 \times 10^8$ CFU per 60-ml central unit). DIF, ENR, or MAR then was injected into the central unit. All experiments were conducted in duplicate with 2-week intervals. The reliability of FQ pharmacokinetic simulations and the high reproducibility of the time-kill curves provided by the model have been demonstrated previously using several drug-pathogen combinations (9, 39). Further, the actual bacterial exposure to the FQ was confirmed by validated high-performance liquid chromatography (HPLC) methods as described earlier (16, 22). Pilot studies done using the reference strain and clinical *S.* Typhimurium strains and two (one high and one low) simulated AUC/MIC ratios found that once the target AUC/MIC was achieved, no strain-specific differences in the kill-regrowth kinetics were observed (data not shown). This was in accordance with our previous studies with similar FQs and other bacterial species, including *E. coli* (16). Therefore, we chose the reference strain, which is a wild-type strain with full genomic and phenotypic characterization, to perform the experiments described below.

**Quantification of the antimicrobial effect and susceptibility changes.** Multiple samples of bacterium-containing media were collected from the central compartment, and the number of bacteria was counted throughout the experimental period. The duration of the experiments was defined in each case as the time until FQ-exposed *S.* Typhimurium after the last dose reached the maximum numbers observed in the absence of antibiotic (≥$10^9$ CFU/ml), and the lower limit of accurate detection was $2 \times 10^2$ CFU/ml. To determine changes of susceptibility during treatment, MICs of bacterial cultures sampled from the system were determined at 24, 48, and 72 h and compared with the initial MICs. The stability of resistance was determined by consecutive passaging of *S.* Typhimurium that was exposed to drugs through drug-free agar plates for 5 consecutive days (9, 16, 22, 38, 39).

**Relationship of the antimicrobial effect to the AUC/MIC ratio.** The time-kill data obtained from the present study were converted to the intensity of the antimicrobial effect ($I_E$), which is defined as the area between the control growth and time-kill curves, with a cutoff level used for the regrowth and control growth curves of $10^9$ CFU/ml (16). With each FQ, the $I_E$ versus log($AUC_{24}$/MIC) data were fitted by the Boltzmann function:

$$Y = \frac{Y_{min} - Y_{max}}{1 + \exp\ [(x - x_0)/dx]} + Y_{max} \quad (1)$$

where $Y$ is the $I_E$, $Y_{max}$ and $Y_{min}$ are its maximum and minimum values, respectively, $x$ is the log($AUC_{24}$/MIC) ratio, $x_0$ is the log(AUC/MIC) ratio that corresponds to $Y_{max}/2$, and $dx$ is the width parameter.

**Relationship of the emergence of resistance to the AUC/MIC ratio.** The MIC values measured at the end of drug exposure ($MIC_{final}$) were related to the values measured before exposing the bacterial strains to the 3-day course of FQs ($MIC_{initial}$), and the fold changes in these MICs were integrated with the simulated AUC/MIC or AUC/MPC ratios using a Gaussian type function (16):

$$Y = Y_0 + a \exp\left[-\frac{(x - x_c)^2}{b}\right] \quad (2)$$

where $Y$ is the $MIC_{final}/MIC_{initial}$ ratio, $Y_0$ is the minimum value of $Y$, $x$ is the log(AUC/MIC) or log(AUC/MPC) ratio, $x_c$ is the log(AUC/MIC) or log(AUC/MPC) ratio that corresponds to the maximal value of $MIC_{final}/MIC_{initial}$, and $a$ and $b$ are parameters. The same equation was used to visualize the $I_E$ relationship to resistance.

**Population dynamics of fluoroquinolone treatment.** In our previous studies using several bacterial species, including *E. coli* and *Actinobacillus pleuropneumonia*, we observed the failure of high concentrations of FQs to clear bacteria in continuously flowing cultures (16, 24). Several mechanisms have been proposed to contribute to these phenomena, including the presence of a persister subpopulation that is physiologically refractory to antibiotics, the presence of an antibiotic refractory wall (biofilm) population that is washed out at a lower rate than the planktonic and persister populations, and density-dependent drug decay in which the effective concentration of the drug in the cultures is substantially less than that anticipated from washout-dependent flow alone (19–21). We tested these mechanisms using previously described methods.

The bioassay approach was used to estimate drug decay by estimating the residual FQ concentration as described previously (19, 24). Briefly, medium was taken from the culture vessels every 6 h, centrifuged, and passed through 0.45-$\mu$m filters (Merck Millipore, Darmstadt, Germany). The activity of this medium against the control ATCC 14028 strain was assessed by the broth microdilution method as described above and was compared with the activity of medium containing freshly prepared drug.

Biofilm formation was evaluated by estimation of bacterial cells released from the inside walls of the culture flasks as previously described (19, 24). Briefly, after termination of continuous culture experiments, the medium was removed, the culture vessels were washed by adding an equal volume of phosphate-buffered saline sonicated for 1 min, and the liquid was removed. This procedure was repeated twice, after which an equal volume of MHB II was added. After 5 min of sonication at 56 kHz, the viable cell density was estimated by culturing dilutions of the wash on agar plates.

Further, a previously described mathematical model that predicts the population dynamics of the treated continuous culture was used to assess the contributions of different subpopulations or drug decay to the changes in viable cell density anticipated for continuous culture experiments (19–21, 24).

**Mechanisms of resistance.** To check for target mutations in quinolone resistance-determining regions (QRDRs), genomic DNA was extracted from both clinical isolates and isolates collected from the *in vitro* model, and PCR amplification was carried out using reported methods (40, 41). Sequences were analyzed by Macrogen (Seoul, South Korea). For gene expression analysis of efflux pump and global regulators, RNA was extracted from independent isolates and quantitative real-time PCR was performed as described previously (25). Relative gene expression was calculated using the $2^{-\Delta\Delta C_T}$ method (where the threshold cycle, $C_T$, indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold) (42).

**Statistical analysis and curve fitting.** All statistical analyses were performed using GraphPad InStat (GraphPad Software Inc., San Diego, CA). A simulation program, Berkeley Madonna, was used for population dynamics prediction estimation of the simulations (http://www.eclf.net). Statistical differences between groups were assessed by one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test (version 9.4; SAS Institute, Cary, NC). Differences were considered significant at a $P$ value of $<0.05$.

**Accession number(s).** The sequences determined in this study were deposited in the GenBank database under accession numbers KU325580 to KU325584, KU325564, KU325552, KU325556, and KU325558.

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We have no conflicts of interest to declare.

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