Mutant-prevention concentration and mechanism of resistance in clinical isolates and enrofloxacin/marbofloxacin-selected mutants of *Escherichia coli* of canine origin

Elias Gebru, Myung-Jin Choi, Seung-Jin Lee, Dereje Damte and Seung Chun Park

Laboratory of Pharmacokinetics and Pharmacodynamics, College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, Republic of Korea

The antibacterial activity and selection of resistant bacteria, along with mechanisms of fluoroquinolone resistance, were investigated by integrating the static [MIC or mutant-prevention concentration (MPC)] and *in vitro* dynamic model approaches using *Escherichia coli* isolates from diseased dogs. Using the dynamic models, selected *E. coli* strains and enrofloxacin and marbofloxacin at a range of simulated area under concentration–time curve over a 24 h interval (AUC$_{24}$)/MIC ratios were investigated. Our results indicated increasing losses in susceptibility of *E. coli* upon continuous exposure to enrofloxacin and marbofloxacin *in vitro*. This effect was transferable to other fluoroquinolones, as well as to structurally unrelated drugs. Our results also confirmed an AUC$_{24}$/MIC (AUC$_{24}$/MPC)-dependent antibacterial activity and selection of resistant *E. coli* mutants, in which maximum losses in fluoroquinolone susceptibility occurred at simulated AUC$_{24}$/MIC ratios of 40–60. AUC$_{24}$/MPC ratios of 39 (enrofloxacin) and 32 (marbofloxacin) were considered protective against the selection of resistant mutants of *E. coli*. Integrating our MIC and MPC data with published pharmacokinetic information in dogs revealed a better effect of the conventional dosing regimen of marbofloxacin than that of enrofloxacin in restricting the selection of resistant mutants of *E. coli*. Target mutations, especially at codon 83 (serine to leucine) of *gyrA*, and overexpression of efflux pumps contributed to resistance development in both clinically resistant and *in vitro*-selected mutants of *E. coli*. We also report here a previously undescribed mutation at codon 116 of *parC* in two laboratory-derived resistant mutants of *E. coli*. Additional studies would determine the exact role of this mutation in fluoroquinolone susceptibility, as well as establish the importance of our findings in the clinical setting.

INTRODUCTION

*Escherichia coli* is the predominant cause of urinary tract infections (UTIs) in both dogs and cats, and is also isolated frequently from contaminated wounds, surgical infections of the skin and adjacent soft tissues, and deep canine pyodermas (Booth, 2001; Mueller & Stephan, 2007). Although fluoroquinolones belong to one of the most useful classes of antimicrobial agents used to combat various infections, including those caused by *E. coli*, resistance in canine *E. coli* isolates to a wide range of fluoroquinolones is increasingly reported (Cohn *et al.*, 2003; Ball *et al.*, 2008). Furthermore, the chemical and structural similarities between veterinary- and human-use fluoroquinolones and the close phylogenetic relationships between *E. coli* isolates from dogs and man have raised concerns of cross-resistance and potential spread of resistant zoonotic bacteria (Johnson *et al.*, 2009; Platell *et al.*, 2010).

The problem of antibacterial-drug resistance continues to increase worldwide, in part because the therapeutic concentrations currently used, which block the growth of the majority of susceptible pathogens, are often the very concentrations required to selectively enrich the resistant, mutant portion of the population (Drlica & Zhao, 2007; Roberts *et al.*, 2008). Hence, the ‘mutant-selection
window (MSW) hypothesis has been developed to describe how drug exposures below the mutant-prevention concentration (MPC) may create the selection of resistant bacterial strains, and many recent studies have emphasized the importance of MPC-based dosing strategies to improve therapeutic outcome and restrict the selection of resistant mutants (Drlica & Zhao, 2007).

Several studies have assessed the antibacterial activity of veterinary fluoroquinolones, as well as their ability to select for resistance (Wetzstein, 2005; Boothe et al., 2006; Grobbel et al., 2007; Pasquali & Manfreda, 2007), by determining the MIC and MPC, which are static in vitro parameters. In practice, however, a pharmacodynamic effect in vivo is rather the result of a dynamic exposure of the infective agent to the unbound drug fraction at the relevant effect site and, therefore, a static condition in an in vitro setting can hardly reflect a dynamic situation in a target organ under in vivo conditions (Mueller et al., 2004).

To be clinically useful, the MIC or MPC determined in vitro at constant antibiotic concentrations cannot be used without consideration of the drug’s pharmacokinetic properties (Olofsson et al., 2006). In this regard, dynamic models that mimic antimicrobial pharmacokinetics in vitro have been used to bridge the static determinations of MIC or MPC and the time-course of the antimicrobial effect at continuously changing drug concentrations. These models have been widely applied to human-use fluoroquinolones to describe drug exposures associated with the risk of resistance emergence in various species of bacteria (Zinner et al., 2003; Firsov et al., 2004; Olofsson et al., 2006). These studies have also established the relationship between pharmacokinetic–pharmacodynamic (PK–PD) indices, such as the area under the concentration–time curve (AUC)/MIC or AUC/MPC ratios, and development of resistance, as well as differing potential of fluoroquinolones in preventing selection of resistance. However, there have been very few attempts to employ this in vitro approach with veterinary fluoroquinolones and pathogenic bacteria of animal origin.

In this study, we first evaluated the in vitro activity, in terms of MIC and MPC, of two of the most common fluoroquinolones used in the veterinary area, enrofloxacin and marbofloxacin, against recent E. coli isolates from diseased dogs. Then, for selected strains of E. coli, we used the in vitro dynamic-model approach to determine the bacterial killing and regrowth kinetics, as well as the relationship between pharmacodynamic indices and antibacterial effect or emergence, of resistant mutants of E. coli. Furthermore, in both clinically resistant isolates and laboratory-derived mutants, we studied the mechanisms of resistance related to amino acid changes in gyrA and parC [plasmid-mediated fluoroquinolone resistance (PMQR) genes] and efflux-pump activity.

**METHODS**

**Antimicrobial agents and bacterial strains.** Pure standards of enrofloxacin and marbofloxacin (≥98% purity; Sigma) were used. Stock solutions were prepared weekly according to the manufacturer’s instructions and working solutions were prepared daily by appropriate dilution. In total, 55 E. coli isolates from dogs were used in this study. The isolates were obtained from diagnostic specimens of diseased dogs that visited the veterinary teaching hospital of Kyungpook National University and from sample collections by Gyeongbuk Veterinary Service Laboratory from pet breeders located in Kyungpook province, Korea. All samples were collected in 2006 (n=14) and 2008 (n=41) from adult and juvenile dogs of both sexes. Handling of the pathogen culture and identification were based on standard microbiological procedures (Isenberg, 1995), including API ID 32E biochemical identification (bioMérieux). After proper identification, the first isolate cultures from each animal were preserved in commercial microbial-storage systems (Pro-Lab Diagnostics). Bacteria stored in the beads were reactivated by culturing on appropriate media and used during the experiments.

Inclusion of bacterial strains in the study was based on clinical history, site of isolation and one sample per animal. Samples from animals with a history of antibiotic treatment within the previous 2 weeks were excluded. In total, 23 E. coli strains were isolated from dogs that presented with clinical signs of deep pyoderma in which all animals also harboured the major pyoderma pathogens, including Staphylococcus pseudintermedius. A multiplex PCR with a commercial GeneChaser E. coli Multi kit (RapiGEN) was used to determine whether the eight strains collected from diarrhoeic puppies represent true pathogens (Fig. 1). Of these, three strains obtained from the veterinary hospital were identified as enteropathogenic E. coli (EPEC). Although their clear role in companion animals is yet to be determined, another three strains obtained from the veterinary service laboratory were identified as enterohaemorrhagic E. coli (EHEC). The other two strains did not fall within the five categories of diarrhoeagenic E. coli detectable by the applied PCR assay. The remaining strains used here were derived from dogs with a history of UTIs collected by diagnostic cystocentesis.

**Determination of MIC and MPC.** The MICs of enrofloxacin and marbofloxacin against E. coli strains isolated from dogs and a quality-control strain (E. coli ATCC 25922) were determined in triplicate using the Clinical and Laboratory Standards Institute (CLSI) broth micro-dilution method (CLSI, 2002). The MPC was determined as described elsewhere (Dong et al., 2000; Firsov et al., 2004). Briefly, the
tested micro-organisms were cultured in Mueller–Hinton broth (MHB) and incubated for 24 h. Then, the suspension was centrifuged (at 4000 g for 10 min) and resuspended in MHB to yield a concentration of 10^10 c.f.u. ml^{-1}. The inocula were further confirmed through the serial dilution and plating of 100 µl samples on drug-free medium. A series of agar plates containing known fluoroquinolone concentrations were then inoculated with E. coli (approx. 10^10 c.f.u.). The inoculated plates were incubated for 48 h at 37 °C and screened visually for growth. To estimate the MPC, logarithms of bacterial numbers were plotted against fluoroquinolone concentrations. The MPC was taken as the point where the plot intersected the x-axis, i.e. the lowest fluoroquinolone concentration that inhibited growth completely. All experiments were performed in triplicate. Potency statistics for the MIC of all strains, as well as the MPC and MPC/MIC of susceptible isolates, including ranges and the 50th and 90th percentiles, were generated.

Three representative isolates were selected for further analysis by using in vitro dynamic models. A detailed description of one of these clinical isolates, designated EC 37, with MICs of 0.13 µg ml^{-1} (enrofloxacin) and 0.25 µg ml^{-1} (marbofloxacin) and MPCs of 0.45 µg ml^{-1} (enrofloxacin) and 1.1 µg ml^{-1} (marbofloxacin), is provided below.

**In vitro dynamic model and simulated pharmacokinetic profiles.** A previously described dynamic model (Zinner et al., 2003; Firsov et al., 2004) was used in this study. Single daily doses of marbofloxacin (half-life, 10 h) and twice daily doses of enrofloxacin (half-life, 4.5 h) administered every 12 h for 3 consecutive days were mimicked. The simulated half-lives were consistent with values reported for dogs: 4.1–5.2 h for enrofloxacin and 9.0–10.9 h for marbofloxacin (Frazier et al., 2000; Walker, 2000; Heinon, 2002; Craigmill et al., 2006; Gebru et al., 2009). The model consisted of two connected flasks, one containing fresh MHB and the other with a magnetic stirrer, the central unit, containing the same broth with either a bacterial culture alone (control experiments) or a bacterial culture plus an antimicrobial agent (killing–regrowth experiments). Peristaltic pumps circulated fresh nutrient medium to the flasks and from the central 60 ml unit at a flow rate of 9.2 ml h^{-1} for enrofloxacin and 4.2 ml h^{-1} for marbofloxacin. The system was placed in an incubator at 37 °C. An overnight culture of E. coli was used to inoculate the central compartment. After 2 h incubation, the resulting exponentially growing bacterial cultures reached 10^8 c.f.u. ml^{-1} (6×10^9 c.f.u. per 60 ml central compartment). Then, enrofloxacin or marbofloxacin was injected into the central unit. Five ratios of the AUC over a 24 h dosing interval (AUC_{24 h})/MIC, including the clinically achievable values at the conventional dosing regimen of both drugs, were simulated. The mean ratios of the simulated AUC_{24 h}/MIC varied from 13 to 251 (enrofloxacin) and from 11 to 265 (marbofloxacin) (Fig. 2). These values corresponded to peak concentrations (C_{max}) that equalled the MIC, fell between the MIC and MPC (i.e. within the MSW) or exceeded the MPC. All experiments were performed in duplicate. The actual bacterial exposure to the fluoroquinolones was further confirmed by a validated HPLC method described previously (Frazier et al., 2000).

![Fig. 2. In vitro-simulated pharmacokinetic profiles of enrofloxacin and marbofloxacin (data for 0–72 h are shown). On the right are indicated the AUC_{24 h}/MIC values and the percentage of the dosing interval during which fluoroquinolone concentrations fell within the MSW. Arrows indicate fluoroquinolone dosing.](image-url)
Quantification of the time–kill curves and antimicrobial effect.
Multiple sampling of bacteria-containing medium from the central compartment was performed throughout the observation period. Samples (100 µl) were diluted serially as appropriate, and 100 µl of each was plated onto agar plates. The duration of the experiments was defined in each case as the time after the last dose until antibiotic-exposed bacteria reached the maximum numbers observed in the absence of antibiotic ($\geq 10^9$ c.f.u. ml$^{-1}$). The lower limit of accurate detection was $2 \times 10^2$ c.f.u. ml$^{-1}$.

Based on the time–kill data, the intensity of the antimicrobial effect ($I_E$, defined as the area between the control-growth and time–kill curves; Firsov et al., 2002) was determined from time zero to the time when the effect could no longer be detected, i.e. the time after the last fluoroquinolone dose at which the number of antibiotic-exposed bacteria reached $10^6$ c.f.u. ml$^{-1}$ (cut-off level).

**Relationships of the antimicrobial effect to the AUC$_{24\ h}$/MIC ratio.** For both enrofloxacin and marbofloxacin, the $I_E$ versus $\log_{10}(\text{AUC}_{24\ h}$/MIC) data were fitted by the Boltzmann function:

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

where $Y$ is the $I_E$, $Y_{\max}$ and $Y_{\min}$ are respectively its maximal and minimal values, $x$ is the AUC$_{24\ h}$/MIC ratio, $x_0$ is the AUC$_{24\ h}$/MIC that corresponds to $Y_{\max}/2$, and $dx$ is the width parameter.

Quantification of resistance and its relationship to AUC$_{24\ h}$/MIC or AUC$_{24\ h}$/MPC. To reveal possible changes in the susceptibility of enrofloxacin/marbofloxacin-exposed *E. coli*, precise fluoroquinolone MICs (with starting concentrations of 12, 14, 16, 18 and 20 µg ml$^{-1}$) of bacterial cultures sampled from the model were determined 24, 48 and 72 h after beginning treatment and at the end of the observation period if it was longer than 72 h. The final MIC (MIC$_{\text{final}}$) was then related to the initial value (MIC$_{\text{initial}}$). The stability of resistance was determined by consecutive passaging of drug-exposed *E. coli* onto antibiotic-free agar plates for 5 days.

To relate the increase in the MIC to the simulated AUC$_{24\ h}$/MIC or AUC$_{24\ h}$/MPC, a Gaussian-type function was used:

$$Y = Y_0 + a \exp[-(x-x_0)^2/b]$$  
(2)

where $Y$ is the MIC$_{\text{final}}$/MIC$_{\text{initial}}$ ratio, $Y_0$ is the minimal value of $Y$, $x$ is $\log_{10}(\text{AUC}_{24\ h}$/MIC) or $\log_{10}(\text{AUC}_{24\ h}$/MPC), $x_0$ is $\log_{10}(\text{AUC}_{24\ h}$/MIC) or $\log_{10}(\text{AUC}_{24\ h}$/MPC) that corresponds to the maximal value of MIC$_{\text{final}}$/MIC$_{\text{initial}}$, and $a$ and $b$ are parameters.

Mechanisms of resistance. PCR amplification and direct DNA sequencing of the quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC* of clinically resistant strains (MIC $\geq 4$ µg ml$^{-1}$) and representative single-step mutants collected from the MPC plates or the dynamic models were carried out according to previously described procedures (Oram & Fisher, 1991; Vila et al., 1996). The amplicons were sequenced by Bioneer (Daejeon, Korea) using the same set of PCR primers. Sequences determined in this study have been deposited in GenBank under accession numbers HQ613397–HQ613401.

As several recent studies have indicated an increasing prevalence of PMQR, all fluoroquinolone-resistant *E. coli* strains in this study were also screened for the PMQR genes *qnrA*, *qnrB*, *qnrS*, *qepA* and *aac(6’)-Ib* by by previously described PCR methods (Wang et al., 2008; Minh Vien et al., 2009).

Additionally, the MICs of both drugs against resistant strains and representative single-step mutants of *E. coli* were tested in the presence and absence of an efflux-pump inhibitor (EPI, 20 µg ml$^{-1}$), Phe–Arg–β-naphthylamide (PAN; Sigma), according to a previously described method (Pasquali & Manfreda, 2007). Furthermore, for these strains, the MICs of two other fluoroquinolones commonly used in small animals, difloxacin and orbifloxacin (with or without EPI), as well as other classes of antibacterial agents, including chloramphenicol, erythromycin, gentamicin and tetracycline, were tested as described above.

**RESULTS**

Antibacterial activity

The *in vitro* antibacterial activities of enrofloxacin and marbofloxacin against *E. coli* strains isolated from dogs are presented in Table 1. Comparable activity with MIC$_{50}$ values of 0.03 µg ml$^{-1}$ (enrofloxacin) and 0.06 µg ml$^{-1}$ (marbofloxacin), and MIC$_{90}$ values of 1 µg ml$^{-1}$ (both drugs), was observed. Four isolates showed clinical resistance for both drugs. The MPC values were also comparable, and a slightly narrower MSW for 90 % of the isolates/(MPC/MIC)$_{90}$ for enrofloxacin (6.0) than marbofloxacin (6.7) was obtained.

Time–kill dynamics

Three isolates were selected initially for analysis by *in vitro* dynamic models. As strain-independent killing–regrowth profiles and $I_E$ values were observed for these strains at both intermediate (60–62) and higher (121–133) simulated AUC$_{24\ h}$/MIC ratios of enrofloxacin and marbofloxacin (data not shown), the discussion below focuses on a strain designated EC 37. The time-courses of killing and regrowth of *E. coli* exposed to enrofloxacin or marbofloxacin are shown in Fig. 3. As seen in the upper panel of Fig. 3, the lowest simulated AUC$_{24\ h}$/MIC ratios of enrofloxacin (13.6) and marbofloxacin (11.2), with peak concentrations

<table>
<thead>
<tr>
<th>Potency</th>
<th>Enrofloxacin</th>
<th>Marbofloxacin</th>
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<tbody>
<tr>
<td>MIC (µg ml$^{-1}$)</td>
<td>0.01–8</td>
<td>0.01–16</td>
</tr>
<tr>
<td>R (%)</td>
<td>7.3</td>
<td>7.3</td>
</tr>
<tr>
<td>MPC (µg ml$^{-1}$)</td>
<td>0.02–2</td>
<td>0.03–4</td>
</tr>
<tr>
<td>MPC/MIC</td>
<td>3.5</td>
<td>3</td>
</tr>
</tbody>
</table>

| R (%) | 7.3 | 7.3 |
| MPC (µg ml$^{-1}$) | 0.02–2 | 0.03–4 |
| MPC/MIC | 3.5 | 3 |

*Table 1.* Comparative activity of enrofloxacin and marbofloxacin against *E. coli* strains isolated from dogs

No. of isolates studied was 55 for both enrofloxacin and marbofloxacin. Resistant isolates (R) were not included in MPC or MPC/MIC determinations.
close to the MICs of both drugs, resulted in only slight and transient reductions in bacterial numbers, with bacterial regrowth occurring at the beginning of each dosing interval. The two higher mean AUC$_{24\text{ h}}$/MIC ratios of enrofloxacin (38.0 and 60.8) and marbofloxacin (33.4 and 62.2), where fluoroquinolone concentrations fell in the MSW for 61–63% (enrofloxacin) and 60–81% (marbofloxacin) of the dosing interval, produced more pronounced reduction in bacterial numbers. However, the effects of the third doses were less pronounced than those of the first two doses of both drugs, and regrowth still occurred by the end of each dosing interval. As shown in the bottom two panels of Fig. 3, the highest AUC$_{24\text{ h}}$/MIC ratios of enrofloxacin (121.3 and 250.9) and marbofloxacin (132.9 and 265.3), where drug concentrations exceeded the MPCs for 57–97% (enrofloxacin) and 73–100% (marbofloxacin) of the dosing interval, resulted in the highest reduction in bacterial counts and regrowth occurred only after the third dose of both drugs.

The respective $I_E$ values correlated well with log$_{10}$(AUC$_{24\text{ h}}$/MIC) ratios for both enrofloxacin and marbofloxacin (Fig. 4). The $I_E$–log$_{10}$(AUC$_{24\text{ h}}$/MIC) plots fitted by equation 1 revealed a comparative activity of enrofloxacin and marbofloxacin, in terms of (AUC$_{24\text{ h}}$/MIC)$_{50}$ (64.1 and 65.8) and maximal $I_E$ ($Y_{\text{max}}$) (544.3 and 541.9) values, respectively. The curves were practically parallel except at simulated AUC$_{24\text{ h}}$/MIC ratios between 60 and 130, where enrofloxacin showed a better activity, and hence a higher slope, than marbofloxacin.

**Emergence of resistance**

Exposure of *E. coli* to enrofloxacin and marbofloxacin for 3 consecutive days resulted in increases in MICs of both drugs at simulated AUC$_{24\text{ h}}$/MIC ratios between 30 and 140. With both drugs, these increases were most pronounced after the third dose. Serial subculture of resistant isolates onto antibiotic-free plates revealed no changes in the elevated MICs, showing stable resistance after five subcultures (data not shown). No loss in susceptibility was observed (MIC$_{\text{final}}$/MIC$_{\text{initial}}$, approx. 1) at the lowest and highest simulated AUC$_{24\text{ h}}$/MIC ratios of both drugs. To relate the increases in MIC to AUC$_{24\text{ h}}$/MIC or AUC$_{24\text{ h}}$/MPC of both drugs, the MICs observed at the end of each treatment were normalized to their respective initial MIC values, and fitted by equation 2. As seen in Fig. 5, the MIC$_{\text{final}}$/MIC$_{\text{initial}}$ versus log$_{10}$(AUC$_{24\text{ h}}$/MIC) data showed a good correlation ($r^2$>0.9) with the central point, where the loss in *E. coli* susceptibility reached maximum, at AUC$_{24\text{ h}}$/MIC ratios of 51 (enrofloxacin) and 43 (marbofloxacin). These values also corresponded to the $T_{\text{MSW}}$ (the

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**Fig. 3.** Kinetics of killing and regrowth of *E. coli* exposed to a 3 day course of enrofloxacin and marbofloxacin. Values at the lower right part of each panel indicate the simulated AUC$_{24\text{ h}}$/MIC ratios. Arrows indicate fluoroquinolone dosing.

**Fig. 4.** AUC$_{24\text{ h}}$/MIC-dependent antibacterial effect of enrofloxacin and marbofloxacin against *E. coli* fitted by equation 1. For enrofloxacin, $Y_{\text{max}}$=544.3, $dx$=3.51 and $x_0$=64.1. For marbofloxacin, $Y_{\text{max}}$=541.9, $dx$=2.95 and $x_0$=65.8.
percentage of the dosage interval that a drug concentration falls within the MSW) of $>60\%$ for both drugs. Similar to the AUC$_{24\ h}$/MIC ratio, a good correlation was also observed between the AUC$_{24\ h}$/MPC ratio and the MIC changes (Fig. 6). The estimated minimum AUC$_{24\ h}$/MPC ratios that may protect the selection of resistant mutants of *E. coli* ($\text{MIC}_{\text{final}}/\text{MIC}_{\text{initial}}$, approx. 1) were 38.9 (enrofloxacin) and 31.6 (marbofloxacin).

### Relationships of MPC with pharmacokinetics

The therapeutic usefulness of MPC is dependent on having its value below the attainable serum and tissue drug concentrations after administration of drug doses that are safe for patients (Blondeau et al., 2001). Therefore, we integrated our *in vitro* data with published pharmacokinetic information of enrofloxacin and marbofloxacin in dogs. Pharmacokinetic parameters at clinically recommended lower and higher doses of both drugs, obtained from package inserts or published sources (Craigmill et al., 2006; Walker, 2000), and calculated PK–PD indices are listed in Table 2.

### Mechanisms of resistance

Fluoroquinolone-resistance phenotypes and amino acid substitutions in clinical isolates and *in vitro* selected mutants of *E. coli* are given in Table 3. Of 55 clinical isolates, four were resistant to fluoroquinolones, with MICs ranging from 4 to 8 µg ml$^{-1}$ (enrofloxacin), 4 to 16 µg ml$^{-1}$ (marbofloxacin and difloxacin) and 8 to 16 µg ml$^{-1}$ (orbifloxacin). PCR amplification and sequencing of the QRDRs of *gyrA* and *parC* revealed a substitution of a leucine for serine at codon 83 of *gyrA*, whilst none of the isolates had amino acid changes in *parC*. Determination of MICs in the presence of 20 µg PAN ml$^{-1}$ (an EPI) showed 4- to 8-fold (enrofloxacin) and 2- to 4-fold (marbofloxacin) reductions in MICs of three of four isolates.
resistant isolates, and 2- to 16-fold (difloxacin) and 2- to 8-fold (orbifloxacin) reductions in MICs of all strains.

Three originally susceptible \textit{E. coli} stains (EC 26, EC 37 and EC 45; MICs ranging from 0.03 to 1 μg ml\(^{-1}\)) were used for the \textit{in vitro} dynamic study (Table 3). Exposure to enrofloxacin and marbofloxacin for 3 days resulted in 4- to 16-fold (enrofloxacin) and 2- to 10-fold (marbofloxacin) increases in MICs, depending on the tested strain and simulated AUC\(24\ h/MIC\) ratio. Enrofloxacin/marbofloxacin-selected mutants also had 4- to 16-fold-higher MICs for difloxacin and orbifloxacin than the original strains. The highest MIC changes that resulted in values higher than the resistant breakpoints set by the CLSI were associated with a change in serine 83 to leucine in the \textit{gyrA} gene of most mutants, and a change in alanine to proline at codon 116 of \textit{parC} in one mutant. Most of the mutants also showed reductions in MICs when tested in the presence of an EPI. The rank order of fluoroquinolones for the highest MIC reduction in the presence of an EPI was orbifloxacin (approx. 8.7-fold), followed by difloxacin (approx. 8-fold), enrofloxacin (approx. 4-fold) and marbofloxacin (approx. 2-fold).

Four representative mutants of \textit{E. coli} selected from MPC plates containing the highest fluoroquinolone concentration were tested for the presence of any target mutations or efflux-mediated resistance. Three fully susceptible parent strains (EC 43, EC 44 and EC 48) had no amino acid substitutions, whereas the fourth original strain (EC 49), with MICs of 1 μg ml\(^{-1}\) (enrofloxacin) and 2 μg ml\(^{-1}\) (marbofloxacin), contained a change in serine 83 to leucine in the \textit{gyrA} gene. MPC mutants had 2- to 8-fold higher fluoroquinolone MICs than the parent strains, which resulted in three resistant phenotypes (difloxacin, enrofloxacin and orbifloxacin) and two resistant and one intermediately susceptible phenotype (marbofloxacin), as defined by the CLSI. All resistant mutants had a serine 83 to leucine change in \textit{gyrA}, but none of them contained \textit{parC} mutations. Similar to mutants obtained from the \textit{in vitro} dynamic model, most MPC mutants also showed 2- to 8-fold reductions in fluoroquinolone MICs when tested in the presence of an EPI, the highest reductions being for orbifloxacin and difloxacin.

Both clinically resistant and \textit{in vitro}-selected mutants were also screened for the PMQR genes by PCR amplification using specific primers for \textit{qnrA}, \textit{qnrB}, \textit{qnrS}, \textit{qepA} and \textit{aac(6')-Ib} and initial size-based identification in ethidium bromide-stained agarose gels. However, none of the PMQR genes were detected in any of the tested strains.

### DISCUSSION

The selection of resistance by an antibacterial agent is an important pharmacodynamic characteristic to evaluate, as this can impact the usefulness of the drug in clinical practice (Rybak, 2006). Although a number of previous studies have assessed the issue of drug resistance in veterinary fluoroquinolones, most studies used clinically resistant isolates from companion animals. These may have limitations in providing specific cause–effect relationships between antibiotic exposure and resistance development.
Furthermore, most previous studies using these agents did not address issues such as the effect of dynamic exposure to various concentrations of one drug on the susceptibility of the organism for the same or other agents, and potential differences in the extent to which drugs within the same class are affected by various resistance mechanisms. Thus, this study attempted to address the above issues by applying both static and dynamic in vitro approaches, as well as determining the mechanisms of fluoroquinolone resistance.

Our in vitro studies revealed comparable activities of enrofloxacin and marbofloxacin based on MIC and MPC values, as well as the width of the MSW (MPC/MIC ratio). However, as both MIC and MPC represent static in vitro parameters, their value may not reflect the pharmacodynamic effect in vivo (Mueller et al., 2004). In this regard, in vitro dynamic models that allow direct assessment of the effects of various concentration profiles provide a much more detailed assessment of the PK–PD relationships (Firsov et al., 2000; Olofsson et al., 2006).

In this study, we applied the above approach to evaluating the in vitro activities of enrofloxacin and marbofloxacin at a range of AUC_{24 h}/MIC ratios, including those achievable at the clinically recommended doses of both drugs, against E. coli of canine origin. Consistent with their similar MIC values, the killing–regrowth kinetics and the I_{E2}log_{10}(AUC_{24 h}/MIC) relationship of both drugs were also comparable for most simulated AUC_{24 h}/MIC ratios (Figs 3 and 4). Furthermore, these effects were independent of bacterial strain, as the killing–regrowth profiles and I_{E2} values were comparable for three E. coli strains from the study at both intermediate (60–62) and higher (121–132) simulated AUC_{24 h}/MIC ratios of both drugs (data not shown).

Consistently, quinolone-specific but bacterial strain-independent relationships between I_{E2} and AUC_{24 h}/MIC ratios have been reported with other fluoroquinolone pairs, including gatifloxacin versus ciprofloxacin (Vostrov et al., 2000), moxifloxacin versus levofloxacin (Firsov et al., 2000) and trovafloxacin versus levofloxacin (Peterson et al., 2002), against both Gram-negative and Gram-positive bacteria.

Both AUC/MIC and AUC/MPC ratios have been associated with either increased or reduced susceptibility to fluoroquinolones, and the breakpoints for each individual fluoroquinolone vary. Accordingly, the loss in susceptibil-

### Table 3. Fluoroquinolone-resistance phenotypes and amino acid substitutions in clinical isolates and in vitro-selected mutants of E. coli

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fluoroquinolone MIC (µg ml(^{-1})) without (with) EPI</th>
<th>MIC (µg ml(^{-1})) of other drugs</th>
<th>Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enr Mar Dif Orb</td>
<td>Chl Ery Gen Tet</td>
<td>gyrA parC</td>
</tr>
<tr>
<td>Parent strains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC 26</td>
<td>0.03 0.06 0.13 0.13</td>
<td>2 64 2 0.5</td>
<td>WT WT</td>
</tr>
<tr>
<td>EC 37</td>
<td>0.13 0.25 1 0.5</td>
<td>2 32 1 2</td>
<td>WT WT</td>
</tr>
<tr>
<td>EC 45</td>
<td>1 1 2 2</td>
<td>4 64 1 1</td>
<td>WT WT</td>
</tr>
<tr>
<td>EC 43</td>
<td>0.25 0.5 1 1</td>
<td>2 64 2 4</td>
<td>WT WT</td>
</tr>
<tr>
<td>EC 44</td>
<td>0.5 0.5 2 2</td>
<td>2 64 2 2</td>
<td>WT WT</td>
</tr>
<tr>
<td>EC 48</td>
<td>1 1 2 4</td>
<td>4 128 2 4</td>
<td>WT WT</td>
</tr>
<tr>
<td>EC 49</td>
<td>1 2 4 4</td>
<td>2 64 1 2</td>
<td>WT WT</td>
</tr>
<tr>
<td>Single-step mutants from the dynamic model</td>
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<td></td>
</tr>
<tr>
<td>EC 26a</td>
<td>0.2 (0.1) 0.3 (0.1) 0.5 (0.1) 0.5 (0.1)</td>
<td>8 64 2 0.5</td>
<td>WT ND</td>
</tr>
<tr>
<td>EC 26b</td>
<td>0.3 (0.1) 0.5 (0.3) 0.6 (0.1) 1 (0.2)</td>
<td>8 128 2 4</td>
<td>WT WT</td>
</tr>
<tr>
<td>EC 37a</td>
<td>0.63 (0.3) 0.5 (0.5) 4 (2) 2 (1.3)</td>
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<td>Ser83.Leu WT</td>
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<tr>
<td>EC 37b</td>
<td>2 (0.5) 3 (1.5) 16 (8) 8 (4)</td>
<td>16 256 1 8</td>
<td>Ser83.Leu Ala116.Pro</td>
</tr>
<tr>
<td>EC 45a</td>
<td>4 (2) 4 (4) 8 (4) 8 (6)</td>
<td>8 128 1 8</td>
<td>Ser83.Leu WT</td>
</tr>
<tr>
<td>EC 45b</td>
<td>16 (4) 8 (4) 16 (2) 16 (3.5)</td>
<td>32 512 2 16</td>
<td>Ser83.Leu Ala116.Pro</td>
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<td>Single-step mutants from MPC plates</td>
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<tr>
<td>EC 43a</td>
<td>1 (0.5) 1 (0.5) 2 (0.5) 4 (1.3)</td>
<td>4 64 2 4</td>
<td>WT ND</td>
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<tr>
<td>EC 44a</td>
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<tr>
<td>EC 48a</td>
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<td>4 128 2 2</td>
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<tr>
<td>EC 49a</td>
<td>8 (2) 8 (4) 16 (4) 16 (4)</td>
<td>2 64 2 2</td>
<td>Ser83.Leu WT</td>
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<td>Resistant clinical strains</td>
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<tr>
<td>EC 51</td>
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<tr>
<td>EC 52</td>
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<td>16 256 1 32</td>
<td>Ser83.Leu WT</td>
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Chl, Chloramphenicol; Dif, difloxacin; Enr, enrofloxacin; Ery, erythromycin; Gen, gentamicin; Mar, marbofloxacin; Orb, orbifloxacin; Tet, tetracycline; WT, wild-type; ND, not determined.
ity of enrofloxacin- and marbofloxacin-exposed *E. coli* depended on the simulated $\text{AUC}_{24\ h}/\text{MIC}$ or $\text{AUC}_{24\ h}/\text{MPC}$ ratios (Figs 5 and 6). A 3 day exposure of *E. coli* to enrofloxacin and marbofloxacin at mean $\text{AUC}_{24\ h}/\text{MIC}$ ratios of 40–60 was associated with up to 16-fold (enrofloxacin) and 12-fold (marbofloxacin) higher MICs than the respective values for the original strains. The estimated $\text{AUC}_{24\ h}/\text{MPC}$ values associated with the prevention of mutant selection were 38.9 (enrofloxacin) and 31.6 (marbofloxacin), suggesting a better *in vitro* activity of a given $\text{AUC}_{24\ h}/\text{MPC}$ ratio of marbofloxacin than enrofloxacin to prevent the selection of resistant mutants. As shown in Table 2, the mutant-restrictive $\text{AUC}_{24\ h}/\text{MPC}$ values could be achievable for 50% of the mutant subpopulation ($\text{AUC}_{24\ h}/\text{MPC}_{50}$) with the conventional dosing regimen of both drugs, with $>2$-fold higher (lower clinical doses) and $>3$-fold higher (higher clinical doses) values for marbofloxacin than enrofloxacin. However, the clinical doses of both drugs were far from reaching the values required for 90% of the mutant subpopulation ($\text{AUC}_{24\ h}/\text{MPC}_{90}$).

We also compared our MPC data with the $C_{\text{max}}$ values achievable at the clinical doses of both drugs in dogs (Table 2). The $\text{MPC}_{50}$ values of both drugs were lower than the respective $C_{\text{max}}$ concentrations achievable at both lower and higher clinical doses. However, attaining $C_{\text{max}}$ values higher than the $\text{MPC}_{90}$ of *E. coli* isolates was possible only with the higher doses of both drugs. Maintaining drug concentrations above the $\text{MPC}_{50}$ ($T>\text{MPC}_{50}$) for the whole dosing interval of 24 h was possible with both clinical doses of marbofloxacin, whereas $T>\text{MPC}_{50}$ of approximately 46 and 88% of the dosing interval of 24 h could be achievable at the lower and higher doses of enrofloxacin, respectively. Only higher doses of both drugs could maintain concentrations above the $\text{MPC}_{50}$ ($T>\text{MPC}_{50}$), with an approximately 3-fold longer duration with marbofloxacin than enrofloxacin.

The above findings may suggest a better activity of the conventional dosage regimen of marbofloxacin than that of enrofloxacin in restricting the selection of resistant mutants. Although it is not globally available at this time, a dual-targeting (both topoisomerase IV and DNA gyrase), third-generation fluoroquinolone, pradofloxacin, has shown superior activity in terms of both lower MPC values than other veterinary fluoroquinolones (Wetzstein, 2005) and clinical efficacy against various infections, including UTIs in cats (Lister *et al.*, 2007) and canine pyoderma (Mueller & Stephan, 2007). It has already been suggested that the introduction into the veterinary market of such agents that combine high therapeutic efficacy with a high potential for restricting the selection for fluoroquinolone resistance would promote rational antibacterial-drug therapy in companion animals (Wetzstein, 2005; Lister *et al.*, 2007).

The presence of mutations in the QRDR of the DNA gyrase enzyme is the primary cause of high-level fluoroquinolone resistance in Gram-negative bacteria such as *E. coli* (Ruiz, 2003). The most frequent mutation observed in quinolone-resistant *E. coli* is at codon 83 of gyrA. Consistently, all clinically resistant isolates and most laboratory-derived mutants of *E. coli* in this study possessed a point mutation in this codon. In addition, mutations in parC, usually at Ser-80, Gly-78 and Glu-84, contribute to high-level fluoroquinolone resistance in clinical *E. coli* isolates (Vila *et al.*, 1996; Ruiz, 2003). However, none of the clinically resistant or *in vitro*-selected mutants of *E. coli* had mutations in the above codons of parC. Rather, two enrofloxacin-selected resistant mutants of *E. coli* possessed a proline for alanine substitution at codon 116 of parC, in addition to a leucine for serine substitution at codon 83 of gyrA. This is, to our knowledge, the first report of mutations at codon 116 of parC in *E. coli*. However, mutations at a similar codon (alanine to proline or glutamic acid) have been reported in *Staphylococcus aureus* (Ng *et al.*, 1996; Ince & Hooper, 2001).

Constitutive and inducible efflux is a known mechanism of fluoroquinolone resistance in both Gram-negative and Gram-positive bacteria (Martínez *et al.*, 2006). In *E. coli*, overexpression of the AcrAB-ToIC system was reported by many investigators to cause multi-drug resistance, including to fluoroquinolones (Poole, 2000). Similarly, analysis of clinically resistant isolates and *in vitro*-selected mutants of *E. coli* in this study revealed a possible involvement of efflux-mediated resistance. Most original strains used in our *in vitro* experiments were efflux-negative, as evidenced by the absence of or only minimal (≤2-fold) reductions of MICs when tested in the presence of an EPI (data not shown). However, many-fold increases in MICs of these strains after exposure to enrofloxacin/marbofloxacin were associated with PAN-sensitive efflux overexpression. Despite the presence of target mutations, mainly at codon 83 of gyrA, the MICs of most resistant strains (both clinical and laboratory-derived strains) decreased in the presence of PAN to levels that rendered them fully or intermittently susceptible to fluoroquinolones.

Our study also revealed that PAN-induced reductions in MICs varied depending on the drug. Orbifloxacin and difloxacin showed the highest reduction in MICs in the presence of PAN, and marbofloxacin was the least affected by PAN-sensitive efflux. Consistent with our findings, a previous study using veterinary fluoroquinolones and *Pseudomonas aeruginosa* demonstrated that the more lipophilic fluoroquinolones, such as difloxacin, are more affected by the overexpression of efflux pump than the less lipophilic ones, such as marbofloxacin (Tejedor *et al.*, 2003).

Fluoroquinolone-efflux systems are believed to have broad substrate specificity in which strains expressing efflux-mediated quinolone resistance show cross-resistance to a number of structurally unrelated antimicrobial agents (Poole, 2000). To confirm this, we analysed the MICs of tetracycline, gentamicin, chloramphenicol and erythromycin against certain enrofloxacin/marbofloxacin-selected...
E. coli mutants, along with the parent strains. Compared with the original strains, mutants had 2- to 16-fold (tetracycline), 4- to 8-fold (chloramphenicol) and 2- to 8-fold (erythromycin) higher MICs, whilst no or ≤ 2-fold differences were observed with the highly hydrophilic agent gentamicin (Table 3).

In conclusion, our findings revealed increasing losses in susceptibility of E. coli upon continuous exposure to enrofloxacin and marbofloxacin in vitro. This effect was transferable to other fluoroquinolones, as well as to structurally unrelated drugs. Our results also confirmed AUC$_{24}$/MIC (AUC$_{24}$/MPC)-dependent antibacterial activity and selection of resistant E. coli mutants. Integrating our MIC and MPC data with published pharmacokinetic information in dogs revealed a better effect of the conventional dosing regimen of marbofloxacin than that of enrofloxacin in restricting the selection of resistant mutants of E. coli. Target mutations, especially at codon 83 of gyrA, and overexpression of efflux pumps contributed to resistance development in both clinically resistant and in vitro-selected mutants of E. coli. We also report here a mutation at codon 116 of parC in two laboratory-derived resistant mutants of E. coli. Additional studies would determine the exact role of this mutation in fluoroquinolone susceptibility, as well as establish the importance of our findings in the clinical setting.

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