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

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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$_{24 h}$)/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$_{24 h}$/MIC (AUC$_{24 h}$/MPC)-dependent antibacterial activity and selection of resistant *E. coli* mutants, in which maximum losses in fluoroquinolone susceptibility occurred at simulated AUC$_{24 h}$/MIC ratios of 40–60. AUC$_{24 h}$/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 resist-ant, 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 ($\geqslant 98\%$ 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
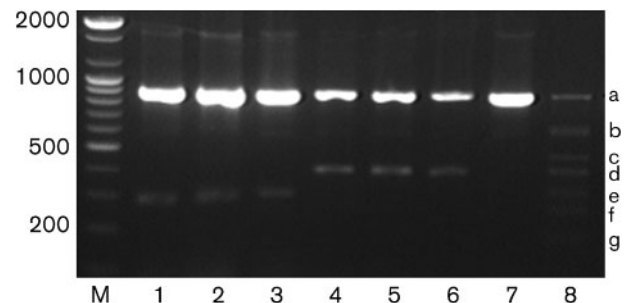

Fig. 1. Multiplex PCR of *E. coli* strains from diarrhoeic dogs. Lanes: M, 100 bp DNA ladder (sizes are shown in bp); 1–3, EPEC; 4–6, EHEC; 7, negative control; 8, positive size markers for internal control (a), enteroinvasive *E. coli* (SPA, b), enterotoxigenic *E. coli* (ETEC-LT, c), EHEC-VT2 (d), EPEC-eae (e), EHEC-VT1 (f) and ETEC-ST (g).

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; Heinen, 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).
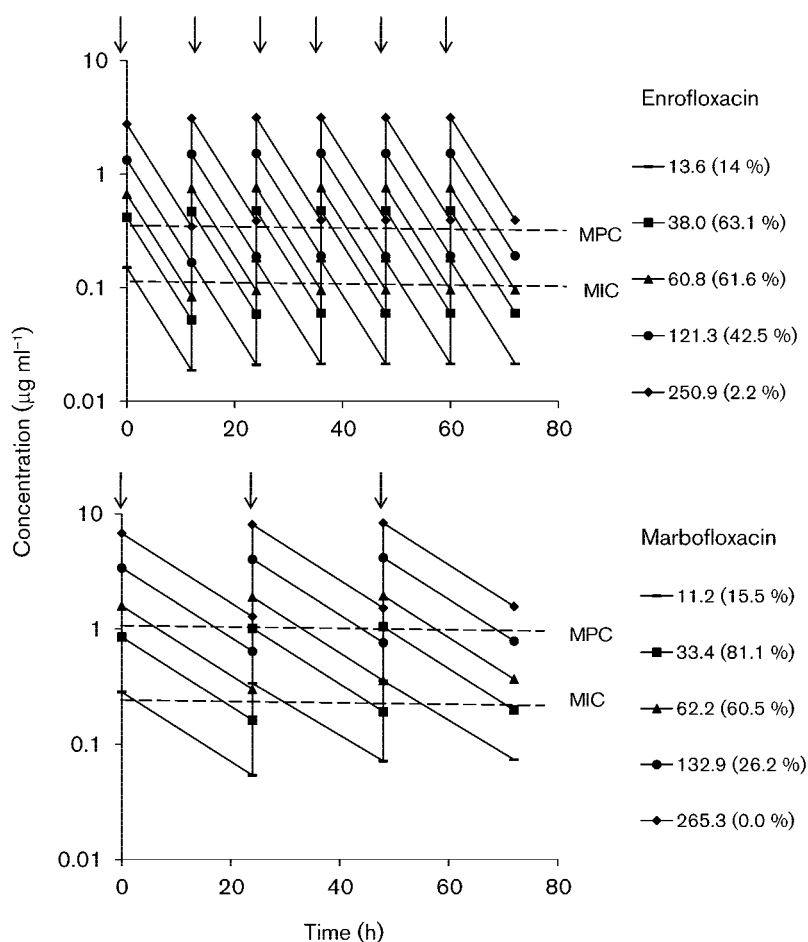

Fig. 2. *In vitro*-simulated pharmacokinetic profiles of enrofloxacin and marbofloxacin (data for 0–72 h are shown). On the right are indicated the AUC$_{24 h}$/MIC values and the percentage of the dosing interval during which fluoroquinolone concentrations fell within the MSW. Arrows indicate fluoroquinolone dosing.

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 ($\geqslant 10^9$ c.f.u. ml^{-1}). The lower limit of accurate detection was 2×10^2 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 10^9 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 $\log_{10}(AUC_{24\,h}/MIC)$ data were fitted by the Boltzmann function:

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

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_{final}) was then related to the initial value ($MIC_{initial}$). 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:

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

where Y is the $MIC_{final}/MIC_{initial}$ ratio, Y_0 is the minimal value of Y, x is $\log_{10}(AUC_{24\,h}/MIC)$ or $\log_{10}(AUC_{24\,h}/MPC)$, x_c is $\log_{10}(AUC_{24\,h}/MIC)$ or $\log_{10}(AUC_{24\,h}/MPC)$ that corresponds to the maximal value of $MIC_{final}/MIC_{initial}$, 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 $\geqslant 4$ 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 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 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.

Potency	Enrofloxacin	Marbofloxacin
MIC (μg ml^{-1})		
Range	0.01–8	0.01–16
MIC_{50}	0.03	0.06
MIC_{90}	1	1
R (%)	7.3	7.3
MPC (μg ml^{-1})		
Range	0.02–2	0.03–4
MPC_{50}	0.27	0.2
MPC_{90}	3.5	3
MPC/MIC		
Range	1.5–10	2–11.7
$(MPC/MIC)_{50}$	3.5	3.3
$(MPC/MIC)_{90}$	6.0	6.7

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 $AUC_{24\,h}/MIC$ 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 $AUC_{24\,h}/MIC$ 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 $\log_{10}(AUC_{24\,h}/MIC)$ ratios for both enrofloxacin and marbofloxacin (Fig. 4). The $I_E - \log_{10}(AUC_{24\,h}/MIC)$ plots fitted by equation 1 revealed a comparative activity of enrofloxacin and marbofloxacin, in terms of $(AUC_{24\,h}/MIC)_{50}$ (64.1 and 65.8) and maximal I_E (Y_{max}) (544.3 and 541.9) values, respectively. The curves were practically parallel except at simulated $AUC_{24\,h}/MIC$ 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 $AUC_{24\,h}/MIC$ 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 ($MIC_{final}/MIC_{initial}$, approx. 1) at the lowest and highest simulated $AUC_{24\,h}/MIC$ ratios of both drugs. To relate the increases in MIC to $AUC_{24\,h}/MIC$ or $AUC_{24\,h}/MPC$ 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 $MIC_{final}/MIC_{initial}$ versus $\log_{10}(AUC_{24\,h}/MIC)$ data showed a good correlation ($r^2 > 0.9$) with the central point, where the loss in *E. coli* susceptibility reached maximum, at $AUC_{24\,h}/MIC$ ratios of 51 (enrofloxacin) and 43 (marbofloxacin). These values also corresponded to the T_{MSW} (the
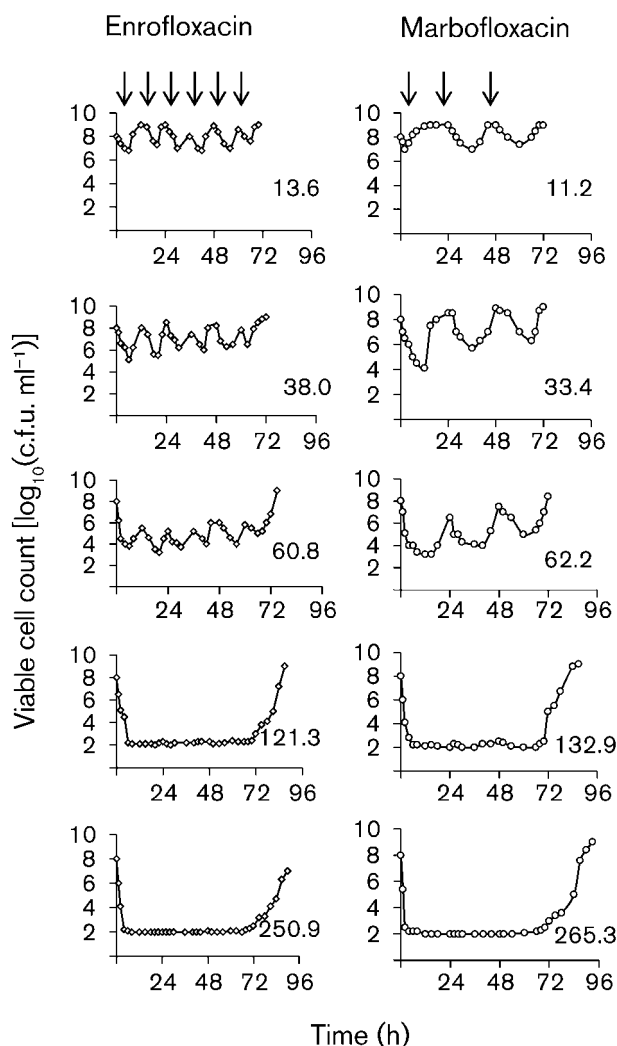

Fig. 3. Kinetics of killing and regrowth of *E. coli* exposed to a 3 day course of enrofloxacin and marbofloxacin. Values at the lower right part of each panel indicate the simulated $AUC_{24\,h}/MIC$ ratios. Arrows indicate fluoroquinolone dosing.
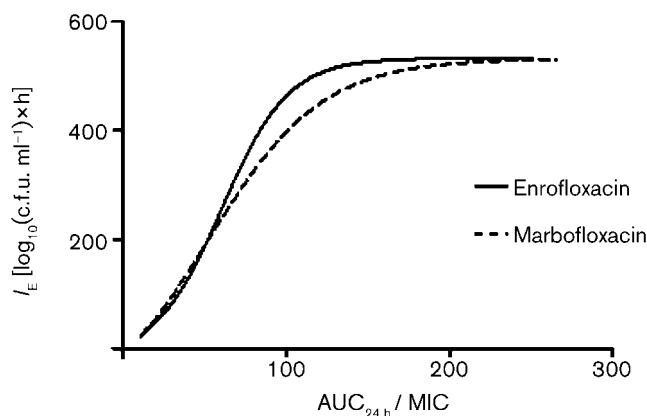

Fig. 4. $AUC_{24\,h}/MIC$-dependent antibacterial effect of enrofloxacin and marbofloxacin against *E. coli* fitted by equation 1. For enrofloxacin, $Y_{max} = 544.3$, $dx = 3.51$ and $x_0 = 64.1$. For marbofloxacin, $Y_{max} = 541.9$, $dx = 2.95$ and $x_0 = 65.8$.
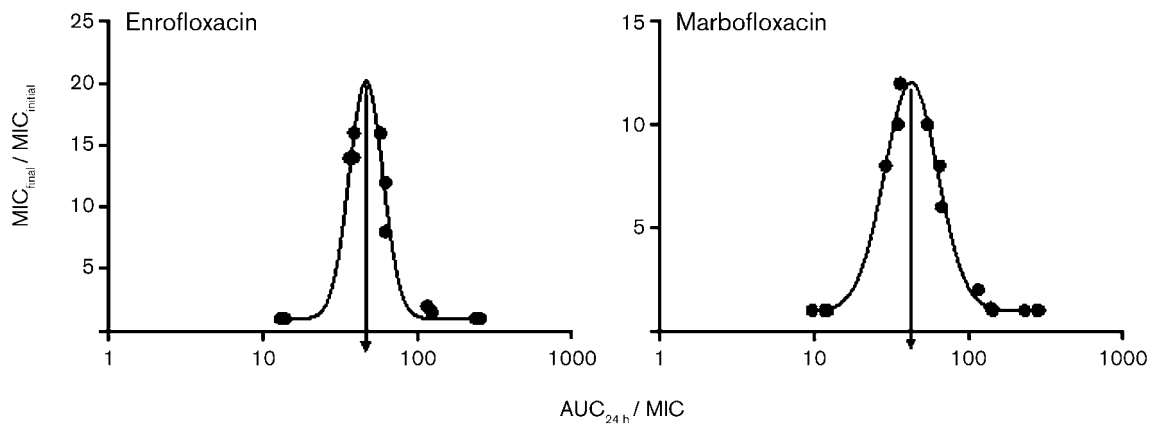
Fig. 5. Effect of $AUC_{24 h}/MIC$ on the susceptibility of *E. coli* exposed to enrofloxacin and marbofloxacin, fitted by equation 2. For enrofloxacin, $a=19$, $x_c=1.71$ and $b=0.15$. For marbofloxacin, $a=11$, $x_c=1.63$ and $b=0.25$.

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* ($MIC_{final}/MIC_{initial}$, 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
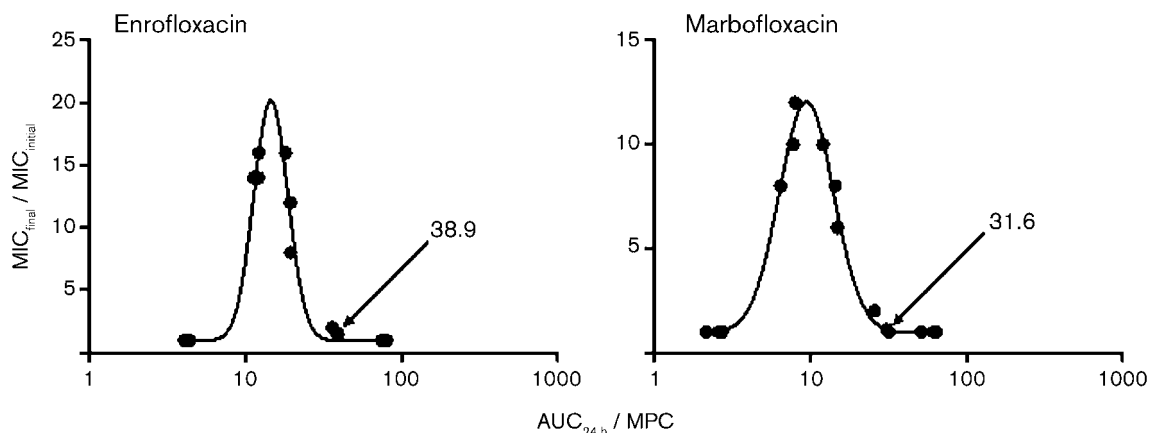

Fig. 6. Effect of $AUC_{24 h}/MPC$ on the susceptibility of *E. coli* exposed to enrofloxacin and marbofloxacin, fitted by equation 2. For enrofloxacin, $a=19$, $x_c=1.16$ and $b=0.15$. For marbofloxacin, $a=11$, $x_c=0.97$ and $b=0.25$.

Table 2. Pharmacodynamic predictors of fluoroquinolone activity based on reported pharmacokinetic data and MIC and MPC of *E. coli* strains from this study

C_{max}, T_{max} (time for maximum observed serum concentration) and $T_{1/2}$ (elimination half-life) values were based on prescription information and published sources given in the text. $T{>}MIC$ and $T{>}MPC$ computations were based on a 24 h dosing interval for both drugs.

	Low dose		High dose	
	Enrofloxacin	Marbofloxacin	Enrofloxacin	Marbofloxacin
Dose (mg kg^{-1})	5.0	2.75	20.0	5.5
AUC (µg h ml^{-1})	10.5	31.0	34.0	64.0
C_{max} (µg ml^{-1})	1.6	2.0	4.2	4.2
T_{max} (h)	1.8	1.8	3.6	1.8
$T_{1/2}$ (h)	4.1	9.1	5.2	10.9
AUC/MIC$_{50}$	350.0	516.7	1133.3	1066.7
AUC/MIC$_{90}$	10.5	31.0	34.0	64.0
AUC/MPC$_{50}$	38.9	155.0	125.9	320.0
AUC/MPC$_{90}$	3.0	10.3	9.7	21.3
C_{max}/MIC$_{50}$	53.3	33.3	70.0	70.0
C_{max}/MIC$_{90}$	1.6	2.0	4.2	4.2
C_{max}/MPC$_{50}$	5.9	10.0	21.0	21.0
C_{max}/MPC$_{90}$	0.46	0.67	1.40	1.40
$T{>}MIC_{50}$ (h)	~24	>24	>24	>24
$T{>}MIC_{90}$ (h)	~3	~9	~11	~23
$T{>}MPC_{50}$ (h)	~11	>24	~21	>24
$T{>}MPC_{90}$ (h)	0	0	~1.5	~5

resistant isolates, and 2- to 16-fold (difloxacin) and 2- to 8-fold (orbifloxacin) reductions in MICs of all strains.

Three originally susceptible *E. coli* stains (EC 26, EC 37 and EC 45; MICs ranging from 0.03 to 1 µg ml^{-1}) were used for the *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 *gyrA* gene of most mutants, and a change in alanine to proline at codon 116 of *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 *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 *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 *gyrA*, but none of them contained *parC* mutations. Similar to mutants obtained from the *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 *in vitro*-selected mutants were also screened for the PMQR genes by PCR amplification using specific primers for *qnrA*, *qnrB*, *qnrS*, *qepA* and *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.

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

Chl, Chloramphenicol; Dfl, difloxacin; Enr, enrofloxacin; Ery, erythromycin; Gen, gentamicin; Mar, marbofloxacin; Orb, orbifloxacin; Tet, tetracycline; WT, wild-type; ND, not determined.

Strain	Fluoroquinolone MIC (µg ml^{-1}) without (with) EPI				MIC (µg ml^{-1}) of other drugs				Mutations	
	Enr	Mar	Dif	Orb	Chl	Ery	Gen	Tet	*gyrA*	*parC*
Parent strains										
EC 26	0.03	0.06	0.13	0.13	2	64	2	0.5	WT	WT
EC 37	0.13	0.25	1	0.5	2	32	1	2	WT	WT
EC 45	1	1	2	2	4	64	1	1	WT	WT
EC 43	0.25	0.5	1	1	2	64	2	4	WT	WT
EC 44	0.5	0.5	2	2	2	64	2	2	WT	WT
EC 48	1	1	2	4	4	128	2	4	WT	WT
EC 49	1	2	4	4	2	64	1	2	Ser83..Leu	WT
Single-step mutants from the dynamic model										
EC 26a	0.2 (0.1)	0.3 (0.1)	0.5 (0.1)	0.5 (0.1)	8	64	2	0.5	WT	ND
EC 26b	0.3 (0.1)	0.5 (0.3)	0.6 (0.1)	1 (0.2)	8	128	2	4	WT	WT
EC 37a	0.63 (0.3)	0.5 (0.5)	4 (2)	2 (1.3)	8	64	1	4	Ser83..Leu	WT
EC 37b	2 (0.5)	3 (1.5)	16 (8)	8 (4)	16	256	1	8	Ser83..Leu	Ala116..Pro
EC 45a	4 (2)	4 (4)	8 (4)	8 (6)	8	128	1	8	Ser83..Leu	WT
EC 45b	16 (4)	8 (4)	16 (2)	16 (3.5)	32	512	2	16	Ser83..Leu	Ala116..Pro
Single-step mutants from MPC plates										
EC 43a	1 (0.5)	1 (0.5)	2 (0.5)	4 (1.3)	4	64	2	4	WT	ND
EC 44a	4 (1)	2 (2)	4 (0.7)	8 (2)	2	128	2	2	Ser83..Leu	WT
EC 48a	4 (4)	4 (2)	16 (2.7)	16 (2.5)	4	128	2	4	Ser83..Leu	WT
EC 49a	8 (2)	8 (4)	16 (4)	16 (4)	2	64	2	2	Ser83..Leu	WT
Resistant clinical strains										
EC 51	4 (4)	4 (4)	4 (2)	8 (4)	4	64	1	2	Ser83..Leu	WT
EC 52	4 (1)	4 (2)	8 (2)	8 (4)	8	128	4	16	Ser83..Leu	WT
EC 53	4 (1)	8 (2)	8 (0.5)	8 (2)	32	128	2	16	Ser83..Leu	WT
EC 54	8 (1)	16 (8)	16 (2)	16 (2)	16	256	1	32	Ser83..Leu	WT

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_E−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_E 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_E 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-

ity of enrofloxacin- and marbofloxacin-exposed *E. coli* depended on the simulated $AUC_{24\,h}/MIC$ or $AUC_{24\,h}/MPC$ ratios (Figs 5 and 6). A 3 day exposure of *E. coli* to enrofloxacin and marbofloxacin at mean $AUC_{24\,h}/MIC$ 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 $AUC_{24\,h}/MPC$ 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 $AUC_{24\,h}/MPC$ ratio of marbofloxacin than enrofloxacin to prevent the selection of resistant mutants. As shown in Table 2, the mutant-restrictive $AUC_{24\,h}/MPC$ values could be achievable for 50 % of the mutant subpopulation ($AUC_{24\,h}/MPC_{50}$) 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 ($AUC_{24\,h}/MPC_{90}$).

We also compared our MPC data with the C_{max} values achievable at the clinical doses of both drugs in dogs (Table 2). The MPC_{50} values of both drugs were lower than the respective C_{max} concentrations achievable at both lower and higher clinical doses. However, attaining C_{max} values higher than the MPC_{90} of *E. coli* isolates was possible only with the higher doses of both drugs. Maintaining drug concentrations above the MPC_{50} ($T>MPC_{50}$) for the whole dosing interval of 24 h was possible with both clinical doses of marbofloxacin, whereas $T>MPC_{50}$ 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 MPC_{90} ($T>MPC_{90}$), 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 (Litster *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; Litster *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 (Martinez *et al.*, 2006). In *E. coli*, overexpression of the AcrAB–TolC 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 ($\leqslant$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-drived strains) decreased in the presence of PAN to levels that rendered them fully or intermediately 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 $\leqslant$ 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 $AUC_{24\,h}/MIC$ $(AUC_{24\,h}/MPC)$-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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