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Mutant prevention concentration and phenotypic and molecular basis of fluoroquinolone resistance in clinical isolates and *in vitro*-selected mutants of *Escherichia coli* from dogs

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ABSTRACT

The antibacterial activity, selection of *Escherichia coli* (*E. coli*) mutants and mechanisms of fluoroquinolone resistance were investigated by integrating the minimum inhibitory concentration (MIC), mutant prevention concentration (MPC) and *in vitro* dynamic model approaches. Difloxacin and orbifloxacin, for which the above information has been scarce, were used. A range of area under curve over a 24 h interval ($AUC_{24\,h}$)/MIC ratios and selected *E. coli* strains were investigated using the dynamic models. Continuous incubation for three days in the presence of difloxacin or orbifloxacin resulted in losses in *E. coli* susceptibility. An $AUC_{24\,h}$/MIC ($AUC_{24\,h}$/MPC)-dependent fluoroquinolone activity and selection of *E. coli* mutants was confirmed. Maximum losses in susceptibility occurred at $AUC_{24\,h}$/MIC ratios of 54 (orbifloxacin) and 57.3 (difloxacin). $AUC_{24\,h}$/MIC ratios of 169.8 (orbifloxacin) and 199.5 (difloxacin) were estimated to be protective against the selection of *E. coli* mutants, and the corresponding ratios based on $AUC_{24\,h}$/MPC predictions were 34 (orbifloxacin) and 36.3 (difloxacin). When integrating our *in vitro* data with pharmacokinetic data in dogs, the conventional clinical doses of both drugs were found to be inadequate to attain the above protective values for 90% of the mutant subpopulation ($AUC_{24\,h}/MPC_{90}$). Both target mutations, esp. at codon 83 (Ser to Leu) of *gyrA*, and overexpression of efflux pumps contributed to resistance development, with mutants also showing decreased susceptibility to enrofloxacin and marbofloxacin. Additional studies would determine the role of mutations found outside the QRDR, at codon 24 of *gyrA*, and at codon 116 of *parC*, and establish the significance of these observations *in vivo*.

1. Introduction

Fluoroquinolones are among the most important drugs used to combat bacterial infections, including those caused by *Escherichia coli* (*E. coli*) in companion animals (Ihrke et al., 1999). However, recent reports from various countries have indicated an increasing resistance in canine *E. coli* isolates to a wide range of veterinary fluoroquinolones (Cohn et al., 2003; Ball et al., 2008). Fluoroquinolone resistance is mediated primarily by target mutations in DNA gyrase (topoisomerase II); with secondary mutations in topoisomerase IV contributing to higher levels of resistance (Yoshida et al., 1990; Vila et al., 1996). Decreased drug uptake due to decreased permeability of the bacterial cell wall or overexpression of energy-dependent efflux pumps also contribute to the development of fluoroquinolone resistance (Ruiz, 2003).

Antibacterial drug resistance is increasing worldwide, in part because the therapeutic concentrations currently used, which are based on the minimum inhibitory

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concentration (MIC) as a measure of potency, are often the very concentrations required to selectively enrich the resistant mutant portion of the population (Drlica and Zhao, 2007; Roberts et al., 2008). Hence, several recent studies have emphasized the importance of mutant prevention concentration (MPC)-based dosing strategies to improve therapeutic outcome and restrict the selection of resistant mutants.

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. By applying these models and using human-use fluoroquinolones, several studies have established the relationship between fluoroquinolone-exposure and the emergence of bacterial resistance (Firsov et al., 2000; Olofsson et al., 2006).

This study was designed to evaluate the *in vitro* activity, in terms of both MIC and MPC of veterinary fluoroquinolones, mainly difloxacin and orbifloxacin, against recent *E. coli* isolates from diseased dogs. Furthermore, for selected strains of *E. coli*, we applied the *in vitro* dynamic model approach to determine the bacterial killing and re-growth kinetics and the relationship between pharmacokinetic–pharmacodynamic (PK–PD) indices versus antimicrobial effect or emergence of *E. coli* mutants. Additionally, we studied the mechanisms of fluoroquinolone resistance by analyzing the mutations in the *gyrA* and *parC* as well as the activity of the efflux pump.

2. Materials and methods

2.1. Antimicrobial agents and bacterial strains

Pure standards of difloxacin, enrofloxacin, marbofloxacin (Sigma–Aldrich, St. Louis, MO) and orbifloxacin (Samyang Anipharm, Seoul, Korea) were used. Stock solutions (1 mg/mL in 0.1% HCl or 0.1% NaOH) were prepared weekly and working solutions were prepared daily by appropriate dilutions with Mueller Hinton Broth (MHB). A total of 54 *E. coli* isolates from skin, gastrointestinal and urinary tract infections of dogs were used. 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 private dog breeders. All samples were collected in 2006 ($n = 14$) and 2008 ($n = 40$) 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. Inclusion of bacterial strains in the study was based on clinical history, site of isolation and one sample per animal basis. Samples from animals with history of antibiotic treatment within the previous two weeks were excluded. A total of 23 *E. coli* strains were isolated from dogs presented with clinical signs of deep pyoderma in which all animals also harbored the major pyoderma pathogens, including *Staphylococcus pseudintermedius*. A multiplex PCR with a commercial GeneChaser *E. coli* Multi Kit (RapiGEN, Gunpo-Si, Korea) was used to determine whether the eight strains collected from diarrheic puppies represent true pathogens. Out 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 enterohemorrhagic *E. coli* (EHEC), and two strains did not fall within the five categories of diarrheagenic *E. coli* detectable by the applied PCR assay. The remaining strains used here were derived from dogs with history of urinary tract infections (UTI) and were collected by diagnostic cystocentesis.

2.2. Determination of MIC and MPC

The MIC of difloxacin and orbifloxacin against all clinical isolates and a quality control strain (*E. coli* ATCC 25922) was determined in triplicate using the Clinical and Laboratory Standards Institute (CLSI) broth micro-dilution method (Clinical and Laboratory Standards Institute, 2002). The MPC was determined as described elsewhere (Dong et al., 2000; Firsov et al., 2003). Briefly, the tested microorganisms were cultured in 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} colony forming unit (CFU)/mL. 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 $\sim 10^{10}$ CFU of *E. coli*. The inoculated plates were incubated for 48 h at 37 °C and screened visually for growth. In addition to two-fold increases of MICs (2×, 4×, 8×, 16×, etc.), more intermediate concentrations (narrow concentration increments) were tested to obtain more precise values of MPCs. All experiments were performed in triplicate and the MPC was defined as the lowest fluoroquinolone concentration that completely inhibited growth after 48 h incubation. To further validate our estimates, a log-linear relationship was assumed in which we plotted the logarithms of bacterial numbers against fluoroquinolone concentrations, and the MPC was taken as the point where the plot intersected the *x*-axis (Firsov et al., 2003). In each determination, six to eight actual data points were used to determine the slope. The description and graphical presentation of the later approach was reported previously (Firsov et al., 2003). As the outcomes of the observed values and values estimated from the curve were naturally comparable, we reported here the observed values of MPCs.

Three representative isolates were selected for further analysis using *in vitro* dynamic models. We used the MIC_{50} values for each collection site (UTI, pyoderma, diarrhea) as a selection criterion for the first 3 isolates (EC13, EC2, and EC37, respectively), one isolate per collection site. A detailed description of one of these clinical isolates, designated as EC13, with an MIC of 0.13 μg/mL (which also represents both the MIC_{50} and modal MICs) for both drugs and an MPC of 0.56 μg/mL (difloxacin) and 0.49 μg/mL (orbifloxacin) is provided below.