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In vitro Pharmacodynamics of Enrofloxacin Against an *Escherichia coli gyrA* Mutant

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Abstract: The aim of this study was to investigate *in vitro* the pharmacodynamics of Enrofloxacin (ENR) against an *Escherichia coli gyrA* mutant (*E. coli* MT/128). Broth microdilution testing was used to determine the Minimum Inhibitory Concentration (MIC) amd multi-step resistance selection was performed until reaching 1 μg mL^{-1} MIC. For time-kill experiments, colony counts were determined by plating each diluted sample onto Plate Count Agar and an integrated pharmacokinetic/pharmacodynamics area measure (log ratio area) was applied to all cfu data. A single C-T mutation was found in gyrA at codon 83. Concentration-dependent bacterial killing was observed for *E. coli* MT/128. Bactericidal activity for this strain was achieved within 4 h at concentrations ≥8 times the MIC with no significant regrowth by 24 h.

Key words: Pharmacodynamic of enrofloxacin, *Escherichia coli*, C-T mutation, significant, microdilution, Turkey

INTRODUCTION

Escherichia coli (*E. coli*) is an important pathogen causing gastrointestinal infections and septicemia in humans and animals and a range of secondary conditions, including respiratory tract infections in animals (Katie *et al*., 2005). The Fluoroquinolones (FQs) have excellent *in vitro* activities against human and animal clinical *E. coli* isolates. However, the number of reports of FQ-resistant *E. coli* strains isolated from humans and animals is increasing (Orden *et al*., 2001). *E. coli*, the most studied microbial model was used because its DNA gyrase-mediated mechanisms of quinolone resistance are likely applicable to a broad range of bacteria (Hooper, 2001).

Quinolones comprise a large group of synthetic antimicrobial agents and are generally divided into four generations depending on their bioavailabilities, distributions and spectra of activities. The drugs, the FQs, exhibit increased activity against the Enterobacteriaceae and other gram-negative bacteria and have some activity against certain gram-positive cocci. The 1st of the FQs approved for use in animals is Enrofloxacin (ENR) (Martinez *et al*., 2006). Quinolones inhibit DNA gyrase and topoisomerase IV by stabilizing the DNA-enzyme complex blocking the progression of the DNA polymerase for replication (Hooper, 2001; Tran and Jacoby, 2002). To preserve effectiveness of FQs in the face of an increasing prevalence of resistant strains, optimal dosing regimens should be implemented with respect to both bactericidal effect and potential selection of resistant strains (Olofsson *et al*., 2007). The bactericidal activity of FQs is dose-dependent amd the goal of a dosing regimen is to maximize the achievable drug concentrations in relation to the Minimum Inhibitory Concentration (MIC) for a pathogen (Kays and Denys, 2001). Effective dosing regimens for such antibiotics requires that either the 24 h AUC/MIC ratio be at least 100-125 against gram-negative bacilli or the C_{max}/MIC ratio of the causative pathogen be >10 (Levison, 2004). The study of pharmacodynamics is central to the optimization of antimicrobial therapy (Owens and Ambrose, 2001). *In vitro* pharmacodynamic models can provide important information about the time course of an antimicrobial effect which can be used in the dose-response relationship and to determine Pharmacokinetic/Pharmacodynamic (PK/PD) target measures that are predictive of clinical efficacy (Craig, 1998; Dalhoff and Schmitz, 2003; Mueller *et al*., 2004; Gloede *et al*., 2010).

Considerable information relating to the optimal use of these agents is available in veterinary medicine, since the FQs are relatively new antimicrobial agents (Coulet *et al*., 2002). Although, various studies have been carried out on the pharmacodynamic properties of FQs (Ross *et al*., 2001; Coulet *et al*., 2002; Zhanel *et al*., 2002), no specific attempt has been made to determine the dose-response relationship of ENR for resistant *E. coli* strains. Thus, the objective of the present study was to investigate *in vitro* the pharmacodynamics of ENR against an *E. coli gyrA* mutant.

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MATERIALS AND METHODS

Evaluation of bacterial susceptibility: Broth microdilution testing was performed to determine the MICs according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS M7-A6). The *E. coli* culture was prepared in Mueller-Hinton Broth (MHB) at 37°C for 16-20 h. ENR (Fluka) was obtained as an analytical standard powder and was dissolved in 20:80 methanol/PBS (v/v). Freshly prepared stock solutions were sterilized using 0.20 μm single-use filter units (Minisart, Sartorius Stedim Biotech.). ENR dilutions ranging from 0.004-256 μg mL^{-1} were prepared in MHB amd inocula with a density equivalent to 0.5 McFarland turbidity were added to tubes containing the ENR dilutions. After incubating at 37°C for 16-20 h, the MICs were defined as the minimum concentration of antibiotic that inhibited growth of the organism. The Optical Densities (ODs) of the cultures were measured at a wavelength of 620 nm.

Multistep resistance selection: Multi-step resistance selection was performed as previously described by Kosowska-Shick *et al.* (2009). Serial passages were performed daily in MHB for *E. coli* ATCC25922 in sub-inhibitory concentrations of ENR. For each sub-sequent daily passage, an inoculum of 1-2 dilutions below the MIC that matched the turbidity of a growth control tube was used. Daily passages were performed until reaching a 1 μg mL^{-1} MIC.

Isolation of total DNA, PCR conditions and sequencing of gyrA: DNA was extracted from bacterial cultures using the Genomic DNA Purification kit (Fermentas) as described by the manufacturer. All extracted DNA samples were stored at -20°C until use.

The genes *gyrA* and *marR* were PCR amplified with specific primers as described previously (Everett *et al.*, 1996). The primers were as follows; *gyrA*-F, 5'-ACGTACTAGGCAATGACTGG; *gyrA*-R, 5'-AGAAGTCGCCGTCGATAGAAC. The PCR mixture (25 μL total volume) contained 0.2 μM of each primer (Alpha DNA), 2.5 μL reaction buffer (Fermentas), 40 μM dNTP mix (Fermentas), 1.25 mM $MgCl_2$ and 1.5 U Taq DNA polymerase (Fermentas). Isolated DNA (1 μL) was used as the template. PCR amplification (Techne TC3000 Thermal Cycler) was performed with the following conditions; initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and a final extension step at 72°C for 10 min. Aliquots (5 μL) were mixed with 2 μL of loading dye (Bio-Rad) and analyzed by electrophoresis on a 1% (w/v) agarose gel using a 100 bp DNA ladder (Fermentas). The gel was stained with ethidium bromide (Vivantis Biochemical). All PCR products were sequenced by Macrogen, Inc. (Korea).

Time-kill experiments: Time-kill experiments were performed as described by Begic *et al.* (2009). A liquid overnight bacterial culture of the mutant strain *E. coli* MT/128 was diluted with MHB and a standard antibiotic stock solution to achieve a starting inoculum of ~10^6 cfu mL^{-1}. The following ENR concentrations were tested; 0, 0.5, 1, 2, 4, 8, 16 and 32 times the MIC. Each 10 mL culture was incubated at 37°C amd samples were withdrawn for the determination of bacterial counts at 0, 1, 2, 4, 6, 8 and 24 h. Colony counts were determined by plating 100 μL of each diluted sample onto Plate Count Agar (Merck).

Pharmacokinetic and pharmacodynamic analyses: An integrated pharmacokinetic/pharmacodynamic area measure (log ratio area) was applied to all cfu data as described by Booker *et al.* (2005). The $AU_{CFU0-24}$ for both growth control and drug containing regimens was normalized by the $AU_{cfu0-24}$ of the growth control and logarithm of this ratio was used to quantify the drug effect as shown in Eq. 1 (Begic *et al.*, 2009):

$$\log \text{ratio area} = \left(\frac{AU_{cfu}\ \text{drug}}{AU_{cfu}\ \text{growth control}} \right) \quad (1)$$

Using non-linear regression, a four-parameter, concentration-effect, Hill-type model was fitted to the effect parameter using OriginLab (Version 8). The inputs were as follows; the dependent variable (E) was log ratio area, E_0 was the measured effect with no drug added, E_{max} was the maximal effect, C:MIC was the concentration of drug divided by MIC, EC_{50} was the C:MIC for which there was 50% maximal effect and H was the Hill constant.

RESULTS AND DISCUSSION

The MIC of ENR for *E. coli* reference strain ATCC 25922 was ≤0.032 μg mL^{-1}. This strain was subjected to daily passages in the presence of sub-inhibitory concentrations of ENR. A resistant strain *E. coli* MT/128 was produced after ENR passage with an increase in MIC from 0.032 to μg mL^{-1}.

The DNA sequences were analyzed using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For gyrA, the *E. coli* MT/128 sequence was compared to that of strain ATCC 11775 (GenBank Accession No.

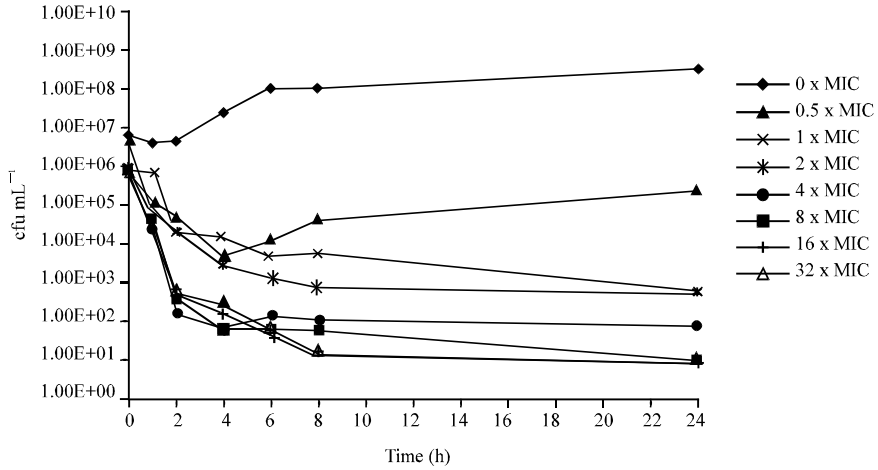

Fig. 1: Time-kill experiments evaluating the bactericidal activity of enrofloxacin against *E. coli* strain MT/128

AF052254) reported by Weigel *et al.* (1998). A single Ct-T mutation was found in gyrA at codon 83, resulting in a Ser83Leu substition. The mutantion was represent in low-level (1 μg mL^{-1}) ENR-resistant *E. coli* and demonstrated typical target site changes. A concentration-dependent bacterial killing was observed for *E. coli* MT/128 with increasing concentrations resulting in greater reduction in bacterial colonies (Fig. 1). Bactericidal activity for *E. coli* MT/128 was achieved within 4 h at concentrations ≥8 times the MIC. The pharmacodynamics of ENR against the gyrA mutant of *E. coli* are shown in Fig. 2. There was a strong correlation between the log ratio area and the C:MIC (R^2 = 0.98). Bacterial inactivation, corresponding to a 4 log reduction was achieved by an ENR concentration of 8 times the MIC within 4 h; no significant regrowth occurred by 24 h. The mutant strain displayed a concentration-effect profile with a Hill's constant of 1.37. The ENR E_{max} and EC_{50} for this strain were 4 and 0.25, respectively.

In recent years, resistance to FQs has been frequently described in clinically isolated *E. coli* in the literature (Drago *et al.*, 2010; Gibson *et al.*, 2010; Bansal and Tandon, 2011).

Therefore, researchers determined the pharmacodynamics of ENR for low-level resistant *E. coli*. In this study, the pharmacodynamics properties of ENR were determined using a log ratio area approach, since this was previously reported as a better fit to the model than the log ratio change approach (Tsuji *et al.*, 2008a, b).

In the current study, the MIC of ENR increased slightly from 0.032-1 μg mL^{-1}. This low level of quinolone resistance in *E. coli* MT/128 resulted from a single mutation in gyrA (Ser83Leu). Low levels of resistance to

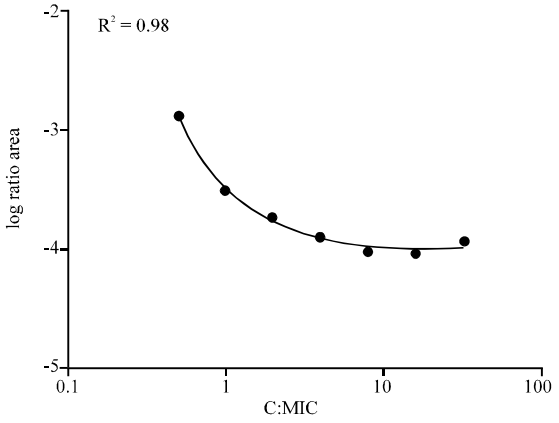

Fig. 2: Pharmacodynamic properties of enrofloxacin against *E. coli* strain MT/128

nalidixic acid and ciprofloxacin were also detected in *E. coli* strains isolated from clinical samples amd the MICs of ciprofloxacin for these strains ranged from 0.125-1.2 μg mL^{-1} (Khan *et al.*, 2005). A single mutation at the ser83 codon in gyrA is also associated with a very low level of resistance to quinolones (Lee *et al.*, 2005).

Dosing regimens are important to minimize the emergence and selection of antibiotic-resistant bacterial strains. The PK/PD measure remains an important predictor of response to antimicrobial therapy and helps to determine the correct dosage (Barger *et al.*, 2003; Booker *et al.*, 2005; Olofsson *et al.*, 2007).

NCCLS (2003) suggests breakpoints for ENR of 0.5 μg mL^{-1} for susceptible microorganisms, 1 μg mL^{-1} for microorganisms of intermediate susceptibility and 2 μg mL^{-1} for those that are resistant (NCCLS, 2001).

However, a single mutation in gyrA attributes to low-level resistance against FQs with a 2-8 fold increase in the MIC (Hooper, 2001). In this study, the pharmacodynamics properties of ENR were described by a Hill-type sigmoidal, maximal effect model, displaying concentration-dependent activity against *E. coli* MT/128; the maximal effect was achieved at higher thresholds of ≥8 times the MIC.

The increase in the PK/PD breakpoint given by this higher dosage suggests that a higher dose should always result in a better efficacy. However, an increase in the dosage usually correlates with increasing efficacy only within a narrow concentration range (Barger *et al*., 2003). Taken together, the results of this study show that the kill rate of the mutant strain did not change by increasing the concentration of ENR from 16-32 μg mL^{-1}.

A Hill-type sigmoidal model can be used to describe the pharmacodynamics of ENR against resistant-*E. coli* with a 32-fold an increase in the MIC resulting in a single gyrA mutation. Development of quinolone resistance can also be predicted using this model since the approach used in the study enables the detection of the correct dosages for bactericidal effect of ENR against resistant *E. coli* strains. Notably, an increase in the dosage causes an increasing efficacy only within a narrow concentration range and the activity of ENR does not change at high concentrations.

CONCLUSION

These results show that increasing in ENR dosage increases its efficacy only within a narrow concentration range amd the activity of this antibiotic does not change at high concentrations.

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