International Journal of Antimicrobial Agents 42 (2013) 544–552

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

Comparative activities of selected fluoroquinolones against dynamic populations of *Actinobacillus pleuropneumoniae* in an in vitro model of time–kill continuous culture experiment

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ARTICLE INFO

Article history:
Received 15 May 2013
Accepted 21 August 2013

Keywords:
Actinobacillus pleuropneumoniae
Marbofloxacin
Enrofloxacin
Difloxacin
Biofilm
Mutations

ABSTRACT

The aim of the current study was to demonstrate and compare the impact of different pharmacokinetics of marbofloxacin, enrofloxacin and difloxacin on their antimicrobial effects, their killing and re-growth kinetics, and the population dynamics of *Actinobacillus pleuropneumoniae* clinical isolates in an in vitro dynamic model. Selected clinical isolates of *A. pleuropneumoniae* and three fluoroquinolones at a range of simulated AUC_{24}/MIC ratios of multiple doses were investigated. At the same simulated AUC_{24}/MIC ratios of the three fluoroquinolones, the killing re-growth profile and I_E values (intensity of the antimicrobial effect) revealed strain- and fluoroquinolone-specific effects. For example, a 31% lower I_E of difloxacin was observed in AppK5 (biofilm-former) than in AppK2 (biofilm-non-former) at the same AUC_{24}/MIC ratio of 120 h. In addition, losses in *A. pleuropneumoniae* susceptibility of both strains by the three fluoroquinolones were observed. AUC_{24}/MPC ratios of 20.89 and 39.81 for marbofloxacin, 17.32 and 19.49 for enrofloxacin and 31.62 and 60.25 for difloxacin were estimated to be protective against the selection of AppK2 and AppK5 strain mutants, respectively. Integration of these in vitro data with published pharmacokinetics revealed the inadequacy of the conventional clinical doses of the three drugs to attain the above protective values for minimum biofilm eradication concentration (MBEC) and concentration to prevent growth of 90% of the mutant subpopulation (MPC_{90}). In conclusion, the results suggest optimising doses could suffice for resistant mutants control, while for biofilm-forming strains combination with biofilm-disrupting agents to reduce the MBEC to achieve AUC/MBEC ratios within the possible dosing regimens is desired.

1. Introduction

Among the major risk factors associated with antibacterial treatment failure include antibacterial drug resistance, which continues to increase worldwide, and the recently rising challenge of biofilm formation by biofilm-forming strains that shields susceptible bacteria from antimicrobials [1–3]. In this regard, the minimum inhibitory concentration (MIC) and the mutant prevention concentration (MPC), in vitro static parameters, have been used in several studies to assess the antibacterial activity of veterinary fluoroquinolones [4–6]. In averting the challenge in antibacterial treatment of biofilm-forming bacterial strains, the use of minimum biofilm eradication concentration (MBEC) is becoming popular, although it is still under pre-clinical investigation as it is difficult to achieve the concentration with current drug regimens [7,8].

The static in vitro pharmacodynamic parameters that have been reported are hardly reflective of the dynamic situation in a target organ under in vivo conditions, which is further augmented by the emerging challenge of eradication of biofilm-forming bacterial strains [9,10]. From this perspective, dynamic models that mimic antimicrobial in vitro pharmacokinetics have been used to bridge the static determinations of MIC/MPC and the time course of the antimicrobial effect at continuously changing drug concentrations. Furthermore, besides mimicking antimicrobial kinetics, the in vitro dynamic model has the additional advantage of studying population dynamics of bacteria in the presence of antibiotics and the possible contributions of the different subpopulation in the antibacterial effect [11].

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http://dx.doi.org/10.1016/j.ijantimicag.2013.08.011

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a severe and highly contagious respiratory disease, with recent reports of antimicrobial resistance in many countries associated with recurrence of respiratory disease in swine farms worldwide [1,12].

Hence, in the current study the in vitro activity, in terms of MIC, MPC and MBEC, of three common fluoroquinolones used in veterinary area (marbofloxacin, enrofloxacin and difloxacin) was first evaluated against recent clinical isolates of *A. pleuropneumoniae* from pigs. Then, for selected biofilm-non-forming and biofilm-forming strains of *A. pleuropneumoniae*, an in vitro dynamic approach was used to determine the bacterial killing and re-growth kinetics as well as the dynamics of the bacterial population in the presence of the three fluoroquinolones and to study the relationship between pharmacokinetic/pharmacodynamic (PK/PD) indices versus antimicrobial effect or the emergence of *A. pleuropneumoniae* mutants and released bacteria from the biofilm. Released bacteria from the biofilm were also estimated and possible mutations in *gyrA*, *gyrB*, *parC* and *parE* genes of isolates from the in vitro dynamic model were also estimated with changes in MICs from the initial MIC. Furthermore, the effects of different subpopulations in the killing re-growth kinetics were studied using a mathematical model with biofilm-non-forming and biofilm-forming *A. pleuropneumoniae* hypothetical strains.

2. Materials and methods

2.1. Antibacterials and bacterial strains

Pure standards of marbofloxacin, enrofloxacin and difloxacin (Sigma–Aldrich, St. Louis, MO) 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 dilution in brain–heart infusion (BHI). A total of 12 clinical isolates originating from respiratory tract infections in pigs as well as the quality control strain *A. pleuropneumoniae* ATCC 27090 were used in this study. Identification of isolates was performed as previously described by Kim et al. [12].

2.2. Minimum inhibitory concentration and mutant prevention concentration determination and minimum biofilm eradication concentration estimation

The MICs of fluoroquinolones against all clinical isolates and a quality control strain (*A. pleuropneumoniae* ATCC 27090) were determined in triplicate using the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method [13]. The MPC was determined as described elsewhere [14,15].

The MBEC was determined using a Calgary Biofilm Device (CBD) (Innovotech, Calgary, Canada) following the manufacturer's instructions. The interpretative criteria of clinical breakpoints were taken from CLSI document M31-A3 and published data.

2.3. Study design

To establish the optimum duration of treatment and dosage regimen, daily dosing of the three fluoroquinolones was simulated for three consecutive days. The 3-day courses of fluoroquinolone administration were simulated over a range of area under the concentration–time curve over 24 h/MIC (AUC_{24}/MIC) ratios (15 h to 250 h). The mono-exponential profiles of pharmacokinetics that mimic once-daily administration of the three fluoroquinolones were simulated with half-lives ($t_{1/2}$) of 8 h for marbofloxacin, 9 h for enrofloxacin and 12 h for difloxacin [16–19] (Fig. 1). Furthermore, a previously described mathematical model [11] of antibiotic treatment with density-dependent and resource concentration-dependent pharmacodynamics was used to predict the relationship between the concentration of each of the three antibiotics and the rate of growth or death of the different bacterial populations.

2.4. In vitro dynamic model

A previously described modified continuous culture device (dynamic model) was used in this study [14,20]. Briefly, the model consisted of two-armed, water-jacketed connected flasks, one containing fresh BHI with 5% β-nicotinamide adenine dinucleotide (β-NAD) and the other with a magnetic stirrer, and the central unit containing the same broth with either a bacterial culture alone (control) or a bacterial culture plus an antimicrobial agent (killing re-growth experiments). Peristaltic pumps circulated fresh nutrient medium to and from the central 60-mL unit. Experiments were not initiated until the densities of bacteria in successive samples were equal and these cultures were exponentially growing and reached ca. 10^8 CFU/mL. Then, marbofloxacin, enrofloxacin or difloxacin was injected into the central unit. All experiments were performed in duplicate within a 1-week interval.

2.5. 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 were serially diluted as appropriate and 100 mL was plated onto agar plates. Based on time–kill data, the intensity of the antimicrobial effect (I_E) was determined from time zero to the time when the effect could no longer be detected [15].

2.6. Relationships of the antimicrobial effect to the AUC_{24}/MIC ratio

For both drugs, the I_E versus log AUC_{24}/MIC data were fitted by the Boltzmann function:

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

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

2.7. Quantification of resistance and its relationship to AUC_{24}/MIC or AUC_{24}/MPC

To reveal possible changes in the susceptibility of *A. pleuropneumoniae* exposed to the three fluoroquinolones, precise fluoroquinolone MICs of bacterial cultures sampled from the model were determined at 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 subculture on antibiotic-free agar plates for 5 days. To relate the increase in the MIC to the simulated AUC_{24}/MIC or AUC_{24}/MPC, a Gaussian type function was used:

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

where Y is the $MIC_{final}/MIC_{initial}$ ratio, Y_0 is the minimum value of Y, x is $\log_{10} AUC_{24}$/MIC or $\log_{10} AUC_{24}$/MPC, x_c is $\log_{10} AUC_{24}$/MIC or $\log_{10} AUC_{24}$/MPC that corresponds to the maximum value of $MIC_{final}/MIC_{initial}$, and a and b are parameters.