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In vitro pharmacokinetics/pharmacodynamics evaluation of marbofloxacin against *Staphylococcus pseudintermedius*

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Abstract: This study aimed at determining the *in vitro* antibacterial activity of a clinically achievable marbofloxacin (MAR) concentration against the clinical isolate *S. pseudintermedius* in an *in vitro* dynamic model simulating the *in vivo* pharmacokinetics of dogs. The *in vitro* PK/PD (pharmacokinetic/pharmacodynamic) model that mimics the single daily doses of MAR (half-life, 8 h) was simulated. An inoculum (10^8 cfu/ml) of clinical isolate *S. pseudintermedius* (MIC = 0.0625 µg/ml) was exposed to monoexponentially decreasing concentrations of MAR with simulated $AUC_{24\,h}$/MIC varied from 34.81 h to 696.15 h. Every two hours, the multiple sample colony forming units were determined. The result of this study demonstrated that the clinically achieved MAR concentrations at $AUC_{24\,h}$/MIC ratios of 348.08 and 696.15 h produced a pronounced reduction in the bacterial counts and prevented the re-growth of the clinical isolate *S. pseudintermedius*. However, further study, considering the strains with different susceptibility levels, is recommended.

Keywords: dogs; clinical isolates; simulation; antimicrobial resistance

S. pseudintermedius is a coagulase positive *Staphylococcus* species which is most commonly associated with canine pyoderma and causes an opportunistic infection in dogs (Morris et al. 2017) and occasionally in humans (Van Hoovels et al. 2006). The *in vitro* and *in vivo* activities of marbofloxacin (MAR), a fluoroquinolone, against *S. pseudintermedius* have been proven in previous studies (Awji et al. 2012). However, the emerging resistances of *S. pseudintermedius* for multiple classes of antimicrobial agents including fluoroquinolones (Awji et al. 2012) could impede the successful treatment outcome in canine pyoderma, especially those caused by prolonged antibiotic treatment (Yoon et al. 2010). Clinically, fluoroquinolones such as MAR should only be used as second-line antibiotics for canine pyoderma to limit the emergence of resistance, which is probably due to the common use of these drugs (Beco et al. 2013).

Since antimicrobial resistance is mainly associated with suboptimal dose at the site of infection (Martinez et al. 2012), an appropriate dose that can minimise the selection of a resistant mutant and maximise the efficacy needs to be achieved. Thus, employment of pharmacokinetic/pharmacodynamic (PK/PD) models have been suggested to select an appropriate dose regimen and to describe the pharmacodynamics information of antimicrobials (Gloede

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et al. 2010). The AUC/MIC, C_{max}/MIC and T > MIC are important PK/PD indices used to predict the efficacy of antimicrobials (Tam and Nikolaou 2011).

A previous *in vitro* static study integrated with published pharmacokinetics parameters suggested that higher doses of MAR within the clinically recommended ranges minimise the selection of resistant mutants (Awji et al. 2012). However, the impacts of the periodic exposure of *S. pseudintermedius* to MAR at clinically achievable concentrations have not been evaluated. In this study, we investigated the activity of MAR against *S. pseudintermedius* in an *in vitro* PK/PD model, using previously suggested doses that minimise the selection of resistant mutants.

MATERIAL AND METHODS

Antimicrobial agent and bacterial strains

In order to obtain the desirable MSW (mutant selection window) for this study, six clinical isolates of *S. pseudintermedius* with the minimum inhibitory concentration (MIC) of MAR at 0.0625 µg/ml were selected as the representative strains from the 52 clinical isolates collected from the dogs which visited the veterinary teaching hospital of Kyungpook National University reported in a previous study (Awji et al. 2012). *Staphylococcus aureus* ATCC 29213 was used as the quality control strain in this study for *Staphylococcus* spp. according to the Clinical and Laboratory Standards Institute (CLSI 2008). A pure standard of MAR (Sigma Aldrich, St. Louis, MO, USA) was used to prepare the stock and working solutions.

Determination of MIC and MPC

The MICs of MAR against *S. pseudintermedius* and *S. aureus* (ATCC 29213) were determined using the broth microdilution method according to the guideline for CLSI (2008). The mutant prevention concentration (MPC) was determined as described elsewhere (Firsov et al. 2003). To estimate the MPC, the logarithms of the bacterial numbers were plotted against the MAR concentrations. The MPC was taken as the lower concentration that completely inhibited growth. All the experiments were performed in triplicate.

In vitro dynamic model simulating *in vivo* pharmacokinetics

A previously described dynamic model (Zinner et al. 2003) was used in this study. A series of monoexponential profiles that mimic the single daily doses of MAR (half-life, 8 h) administered for three consecutive days were simulated. The simulated half-life was obtained from the pharmacokinetic analysis of our study (data not shown) which is closer to the value reported previously by Heinen (2002). The model consisted of two connected flasks, one containing the fresh Mueller Hinton Broth (MHB) and the other, the central unit with a magnetic stirrer, MHB plus either the bacterial culture alone (control) or with the antimicrobial (killing/re-growth experiment). Peristaltic pumps circulated the fresh MHB to the flasks and from the central unit at 8.6 ml/h. An overnight culture of *S. pseudintermedius* was inoculated to the central unit. After a 2-h incubation, the resulting exponentially growing bacterial cultures reached 10^8 colony-forming units per millilitre (cfu/ml) as reported previously (Gebru et al. 2012). Then the MAR was injected into the central unit. Five ratios of an area under the concentration time curve (AUC) over a 24 h dosing interval ($AUC_{24\,h}$/MIC) including the clinically achievable values at the conventional dosing regimen of MAR were simulated and the mean ratios of the simulated $AUC_{24\,h}$/MIC varied from 34.8 h to 696.2 h (Figure 1). These values corresponded to the peak concentrations (C_{max}) that equalled the MIC, which fell between the MIC and MPC (within MSW) or exceeded the MPC. All the experiments were performed in duplicate. The actual bacterial exposure to MAR was further confirmed by the HPLC (high-performance liquid chromatography) analysis described previously (Frazier et al. 2000).

Quantification of the time-kill curves and antimicrobial effect

The multiple sampling of the bacteria containing media from the central compartment was performed throughout the observation period. The colony-forming unit (cfu) was determined for each sample at each sampling time point and the duration of the experiments was defined in each case as the time until the antibiotic-exposed bacte-

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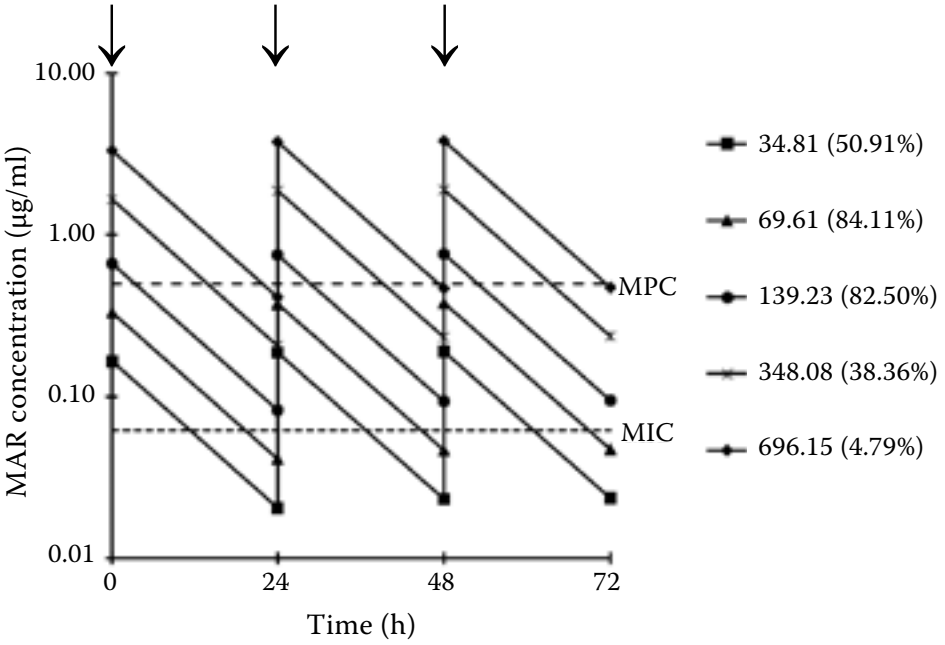

Figure 1. The *in vitro* simulated pharmacokinetic profile of MAR (data for 0–72 h). The legends indicate the AUC_{24h}/MIC values and percentage of the dosing interval during which the MAR concentration fell within the MSW. The arrows indicate the MAR dosing

ria reached the number of bacteria observed in the absence of the antibacterial (≥ 10^9 cfu/ml). The lower limit of accurate detection was 2 × 10^2 cfu/ml. To reveal changes in the susceptibility, the MAR MICs of the bacterial cultures sampled from the model were determined at 24, 48, and 72 h after the treatment begins.

The final MIC (MIC_{final}) was then related to the initial MIC ($MIC_{initial}$). The cumulative effect of each simulated treatment of MAR on the susceptible *S. pseudintermedius* subpopulations was expressed by its intensity (I_E) as described by the area between the control growth and the bacterial killing re-growth curves from the zero point, the moment of the drug input into the model, up to the time when viable counts on the re-growth curve are close to the maximum values observed without the drug (Firsov et al. 2002).

Relationship of the antimicrobial effect to the $AUC_{24\,h}$/MIC ratio

The I_E versus $logAUC_{24\,h}$/MIC data were fitted by the Boltzmann function:

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

where:

Y – I_E;

Y_{max}, Y_{min} – maximum and minimum values of I_E;

x – $logAUC_{24\,h}$/MIC ratio;

x_0 – $logAUC_{24\,h}$/MIC ratio that corresponds to $Y_{max}/2$;

dx – parameter width.

Quantification of the resistance and its relationship to $AUC_{24\,h}$/MIC ratio

The changes in susceptibility of *S. pseudintermedius* for MAR was evaluated at 24, 48 and 72 h time points after beginning the treatment and a Gaussian type function was used to relate the increased MIC to the simulated $AUC_{24\,h}$/MIC or $AUC_{24\,h}$/MPC:

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

where:

Y – $MIC_{final}/MIC_{initial}$ ratio;

x – $logAUC_{24\,h}$/MIC or $AUC_{24\,h}$/MPC;

x_c – $logAUC_{24\,h}$/MIC or $AUC_{24\,h}$/MPC that corresponds to the maximal value of $MIC_{final}/MIC_{initial}$;

a, b – parameters.

Statistical analysis

Statistical analyses were performed using GraphPad InStat (GraphPad Software Inc., San Diego, CA, USA). A simulation program, Berkeley

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Madonna™ was used for the population dynamics prediction estimation of the simulations (http://www.eclf.net).

RESULTS

The pharmacokinetics profile of MAR was simulated in the *in vitro* system (Figure 1). The MIC and MPC identified for *S. pseudintermedius* were 0.0625 and 0.5 µg/ml, respectively. The simulated MAR dose regimens of 10, 20, 40, 100 and 200 µg/ml produced $AUC_{24\ h}$/MIC ratios of 34.89, 69.61, 139.23, 348.07 and 696.15 h, respectively. The simulated $AUC_{24\,h}$/MIC values (69.61 and 139.23 h), with the MAR peak concentration closest to the MPC also have a T_{MSW} (the mutant selection window) of 84.11% and 82.5 %, respectively. The time courses of the viable counts that reflect the killing and re-growth of *S. pseudintermedius* exposed to the monoexponentially decreasing concentrations of MAR are shown in Figure 2. The simulated $AUC_{24\,h}$/MIC values of 69.61 and 139.23 h showed a slightly higher reduction in the viable counts compared to simulated $AUC_{24\,h}$/MIC ratios of 34.89 h (Figure 2). An increase in the MICs of MAR against *S. pseudintermedius* was observed at the simulated concentrations with the $AUC_{24\ h}$/MIC ratios between 71.93 h to 320 h. At the $AUC_{24\,h}$/MIC ratios of 34.81, 69.61 and 139.23 h, the bacterial re-growth was observed during each dosing interval. The simulated $AUC_{24\ h}$/MIC ratio of 696.15 h inhibited the re-growth of the surviving subpopulation until 68 h of the dosing interval.

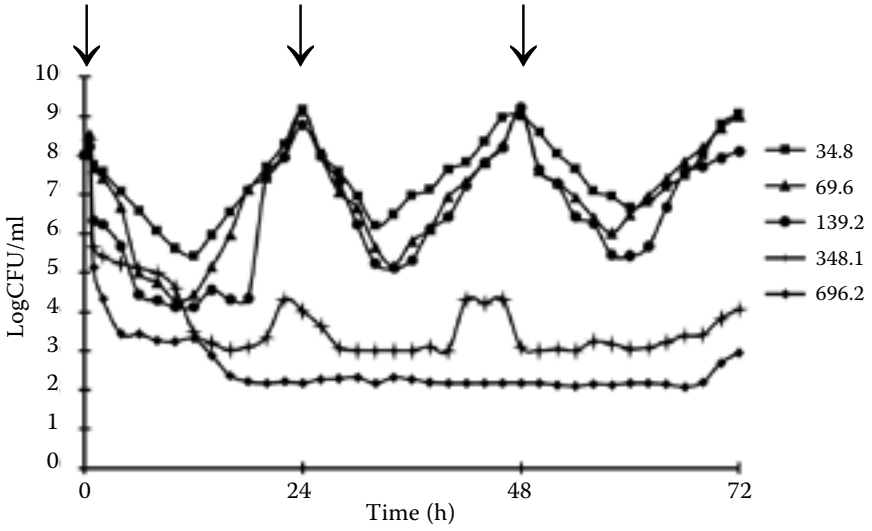

Figure 2. The kinetics of the killing and re-growth of *S. pseudintermedius* exposed to a 3-day course of MAR at different doses. The values stated on the right indicate the simulated $AUC_{24\,h}$/MIC ratios. The arrows indicate the MAR dosing

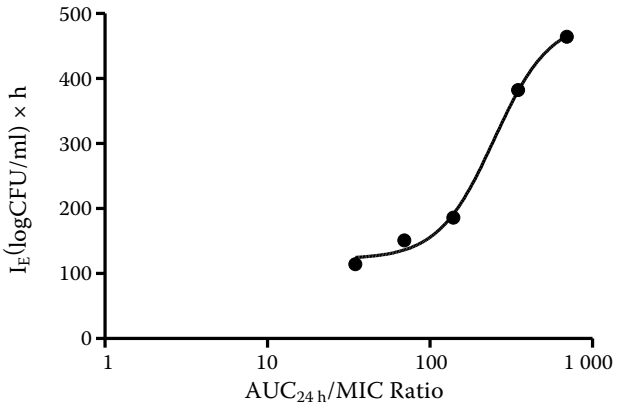

Figure 3. The $AUC_{24\ h}$/MIC dependent antibacterial effect of MAR against *S. pseudintermedius* fitted by Equation 1 (Y_{max} = 490.5, dx = 2.531, x_0 = 2.395 and r^2 = 0.996)

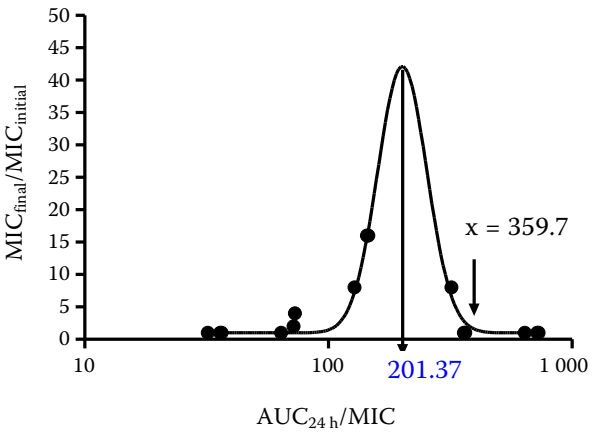

Figure 4. The effect of $AUC_{24\,h}$/MIC on the susceptibility of *S. pseudintermedius* exposed to MAR, fitted by Equation 2 (a = 41.14, x_c = 201.37, r^2 = 0.95, and b = 0.14)

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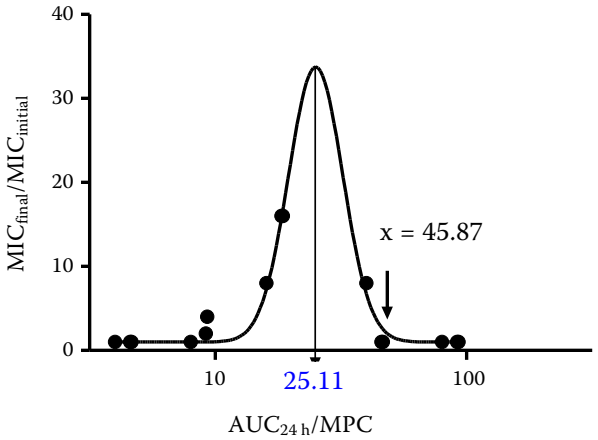

Figure 5. The effect of $AUC_{24\,h}$/MPC on the susceptibility of *S. pseudintermedius* exposed to MAR, fitted by Equation 2 (a = 32.75, x_c = 25.11, r^2 = 0.96, and b = 0.15)

Based on time-kill data obtained, the plot of I_E versus log$AUC_{24\,h}$/MIC for the simulated doses of MAR is presented in Figure 3. The relationship between the antimicrobial activity of MAR and the simulated AUC was fitted by Equation 1. The data showed a good correlation (r^2 = 0.996) between I_E and log$AUC_{24\,h}$/MIC. The simulated $AUC_{24\,h}$/MIC ratio, which could produce 50% maximum antimicrobial activity, was 248.5 h. The minimum and maximum I_E values of the simulated $AUC_{24\,h}$/MIC ratios were between 122.3 and 490.5 h. The slope of the plot of I_E versus log$AUC_{24\,h}$/MIC was 2.531.

No loss in susceptibility was observed (MIC_{final}/$MIC_{initial}$, approximately 1) at the lowest and highest $AUC_{24\,h}$/MIC ratios. In addition, the loss in susceptibility of *S. pseudintermedius* reached a maximum at $AUC_{24\,h}$/MIC ratios of 201.37 h (Figure 4). The $AUC_{24\,h}$/MPC ratio of MAR that restricts the selection of the resistant mutant was 25.11 h (Figure 5).

DISCUSSION

The antibacterial activity of MAR performed with achievable serum concentrations in dogs, revealed an optimised dose that would prevent the change in MIC of the susceptible clinical isolate *S. pseudintermedius*. The simulated $AUC_{24\,h}$/MIC ratios of 348.07 and 696.15 h with peak concentrations above the MPC of *S. pseudintermedius* in this study produced pronounced bacterial killing over the period of the entire dosing interval, see Figure 2. An increased re-growth of *S. pseudintermedius* was observed over the period of the entire treatment period at the simulated $AUC_{24\,h}$/MIC ratios of 34.81, 69.61 and 139.23 h. This re-growth could be due to the monoexponential decrease in the drug concentration over time or the selected mutants due to the drug exposure (Balaban et al. 2004). Also, it might be due to the first step mutant selection as the simulated $AUC_{24\,h}$/MIC ratios fell within the MSW for > 50% of the overall dosing intervals in which the first step mutant was selected (Firsov et al. 2003). The $AUC_{24\,h}$/MIC ratio (30 to 55) of fluoroquinolone used in their study is lower than the $AUC_{24\,h}$/MIC ratio (69.61) in this study and it sufficiently eradicated *Streptococcus pneumonia* (Lacy et al. 1999). However, the susceptibility difference between the bacteria and the difference in the potency of fluoroquinolones are expected.

In this study, simulated doses of MAR above the MIC of *S. pseudintermedius* produce a relatively dose proportional reduction in the viable counts. Similarly, the dose dependent killing rates of fluoroquinolones shown in Figure 2 have been reported in previous studies (Damte et al. 2013; Lee et al. 2017). In addition, the higher correlation (r^2 = 0.99) established between the I_E and $AUC_{24\,h}$/MIC ratio values suggests the concentration dependent killing of MAR (Figure 3). The selection of resistant *S. pseudintermedius* occurred when the MAR concentrations were inside the MSW (T_{MSW}) for > 40% of the dosing interval. Increases in the MIC were observed at the $AUC_{24\,h}$/MIC values that correspond to the MAR concentrations falling into the MSW over most of the dosing interval (T_{MSW} of 40% to 90%). This susceptibility loss might be due to the selected mutants expected within the MSW, which favours the selection of the first step mutants (Gebru et al. 2012). The peak concentrations of MAR at the simulated $AUC_{24\,h}$/MIC ratio of 348.08 h is achievable in dogs (Frazier et al. 2000; Heinen 2002).

A good correlation (r^2 = 0.95) between MIC_{final}/$MIC_{initial}$ and log$AUC_{24\,h}$/MIC or $AUC_{24\,h}$/MPC was obtained (Figures 4 and 5). Our results indicate that a dosing strategy based on an $AUC_{24\,h}$/MPC higher than 45.87 was effective at preventing MAR resistant mutants which was within the range of the $AUC_{24\,h}$/MPC ratios (26.14–70.88) calculated by integrating the PK data in dogs (Frazier et al. 2000; Heinen 2002) and the MPC of our tested strain. Another study of the integration of the

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PK data from a Beagle dog (Heinen 2002) with an MPC_{90} (at approximately 3.9) of MAR against the clinical isolates of *S. intermedius* (Wetzstein 2005) indicated a lower $AUC_{24\ h}$/MPC ratio (26.14) than the estimated $AUC_{24\ h}$/MPC ratios of 45.87 in this study. This suggests that the clinically achievable MAR at 2 mg/kg might not be able to minimise the selection of resistant mutants.

The result of this study demonstrated that clinically achievable MAR concentrations at the simulated $AUC_{24\ h}$/MIC ratios of 348.07 and 696.15 h resulted in the pronounced reduction in the bacterial counts and they prevent the re-growth against the clinical isolate *S. pseudintermedius*. However, further study considering the strains with different levels of susceptibility is recommended.

Conflict of interest

The authors declare no conflict of interest.

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