Evaluation of antimicrobial activity against *Mycoplasma mycoides* subsp. *mycoides* Small Colony using an *in vitro* dynamic dilution pharmacokinetic/pharmacodynamic model

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The objectives of this study were to assess the activity of oxytetracycline (OTC), danofloxacin and tulathromycin against *Mycoplasma mycoides* subsp. *mycoides* Small Colony, the causative agent of contagious bovine pleuropneumonia, in an *in vitro* dynamic concentration model and to determine the concentration and/or time dependence of such activity. Time–kill assays that simulated elimination of antimicrobials from the body were performed. Initial antimicrobial concentrations corresponded to various multiples of the MIC and cultures were diluted in a stepwise fashion with either drug-free or drug-containing artificial medium to mimic administration by single-release bolus or infusion, respectively. Where appropriate, data were fitted to sigmoidal *E*<sub>max</sub> models. OTC produced no change in mycoplasma titre from the initial inoculum size, regardless of the concentration or means of drug exposure. Both danofloxacin and tulathromycin resulted in a decrease in mycoplasma titre but neither was bactericidal (99.9 % kill) over 12 h. A greater antimycoplasmal effect, defined as the change in log<sub>10</sub> (c.f.u. ml<sup>−1</sup>) over 12 h, was achieved when danofloxacin was administered as a single-release bolus, suggesting concentration-dependent activity, whereas the antimycoplasmal effect of tulathromycin was comparable following administration by single-release bolus or infusion, owing to its long half-life.

INTRODUCTION

*Mycoplasma mycoides* subsp. *mycoides* Small Colony (*Mmm*<sub>SC</sub>) is the causative agent of contagious bovine pleuropneumonia (CBPP), a pneumonic disease of cattle in sub-Saharan Africa that has substantial economic importance (Tambi et al., 2006). Control in affected countries currently relies on public sector deployment of live attenuated vaccines. However, these have limited efficacy (Rweyemamu et al., 1995) and state veterinary services are rarely sufficiently resourced to attain the necessary level of vaccination (Mariner et al., 2006a; Tambi et al., 2006). Farmers often have limited confidence in control measures implemented by the public sector and, because they have no access to vaccines, turn instead to antimicrobials to treat their animals. The use of antimicrobials is controversial largely because of the perception that they promote the development of chronic carriers (Provost et al., 1987) but also as a result of concerns regarding the development of resistance. As such, antimicrobials are discouraged or banned in most affected countries (Huebschle et al., 2006).

A number of products have demonstrated efficacy against *Mmm*<sub>SC</sub> *in vitro*, including oxytetracycline (OTC), danofloxacin and tilmicosin (Ayling et al., 2000, 2005). Modelling studies have predicted that antimicrobials, used in association with vaccination, could be successful in eradicating disease from infected herds (Mariner et al., 2006b). Nevertheless, reports on the *in vivo* efficacy against this organism are limited. OTC has been observed to prevent death from CBPP but failed to eliminate the pathogen (Yaya et al., 2004), suggesting that disease transmission would not be prevented. In contrast, whilst danofloxacin had no obvious effect on the course of infection, it appeared to limit the spread of disease to in-contact animals (Huebschle et al., 2006). These results are consistent with only partial efficacy and additional research is clearly required to identify the most appropriate antimicrobial for CBPP control and to define an optimal dosage for its deployment.

Pharmacokinetic/pharmacodynamic (PK/PD) modelling approaches can determine dosage strategies best suited for promoting elimination of bacteria, thus reducing the risk of persistent carrier status and the development of resistance (Lees et al., 2004), and have been used successfully in veterinary medicine (Sarasola et al., 2002). At present, little
information is available on the PK/PD interactions of antimicrobials with known efficacy against MmmsSC. Fixed-concentration models have been used to investigate the activity of OTC, danofloxacin and tulathromycin against MmmsSC in vitro (Mitchell et al., 2011). However, these models do not account for the decline in concentration as drug is cleared from the body. The aim of the current study was to compare the activity of these same antimicrobials against the virulent B237 strain of MmmsSC using an in vitro PK/PD model that simulated elimination. Furthermore, concentration and/or time dependence of antimicrobial activity, which has major implications for how the drug is used under field conditions, were assessed by simulating administration by either single-release bolus or infusion.

METHODS

Materials. MmmsSC strain B237, isolated in Kenya (Jores et al., 2008), was provided by Joachim Frey (University of Bern, Switzerland). OTC hydrochloride and danofloxacin were obtained from Sigma-Aldrich and tulathromycin was supplied by Pfizer. Stock solutions of danofloxacin (2000 mg l\(^{-1}\)) and tulathromycin (1280 mg l\(^{-1}\)) were prepared in 0.01 M sodium hydroxide and 0.0015 M citric acid, respectively, and stored at \(-80^\circ\)C until use. Fresh solutions of OTC hydrochloride were prepared in ddH\(_2\)O at 2000 mg l\(^{-1}\) on each occasion. Drug purity was taken into consideration and drugs were tested with regard to their active base. Controls were performed to demonstrate that the presence of solvents at concentrations used to dissolve antimicrobials had no effect on the growth of MmmsSC.

The MICs for OTC, danofloxacin and tulathromycin against MmmsSC strain B237 were determined previously in custom-made inhibitor-free artificial medium (Mycoplasma Experience) using a macrodilution technique. The values obtained were 0.40, 0.15 and 0.02 mg l\(^{-1}\), respectively, at an inoculum size of 10\(^7\) c.f.u. ml\(^{-1}\) (Mitchell et al., 2011).

Time–kill assays. To prepare the inoculum, mycoplasma was cultured in pre-warmed liquid medium (Mycoplasma Experience) at 37 \(^\circ\)C and diluted while in the exponential phase to give an inoculum of 10\(^7\) c.f.u. ml\(^{-1}\) in final volumes of 2 ml for studies with danofloxacin and 4 ml for those with OTC or tulathromycin. Initial antimicrobial concentrations corresponded to various multiples of the MIC and drugs were added to cultures of mycoplasma to simulate administration by either single-release bolus (1–16 \(\times\) MIC of OTC and danofloxacin, 1–64 \(\times\) MIC of tulathromycin) or infusion (1, 1.5 and 2 \(\times\) MIC of all three antimicrobials, plus 3, 4, 7 and 14 \(\times\) MIC for tulathromycin). Growth controls were included (0 \(\times\) MIC of all drugs) to provide a baseline for comparison of effects of antimicrobials with known efficacy against SC strain B237, isolated in Kenya (Jores et al., 2008). Fixed-concentration models do not account for the decline in concentration as drug is cleared from the body. The aim of the current study was to compare the activity of these same antimicrobials against the virulent B237 strain of MmmsSC using an in vitro PK/PD model that simulated elimination. Furthermore, concentration and/or time dependence of antimicrobial activity, which has major implications for how the drug is used under field conditions, were assessed by simulating administration by either single-release bolus or infusion. 10\(^{-1}\). Aliquots (10 µl) of each dilution were transferred to solid medium (Mycoplasma Experience) and incubated at 37 \(^\circ\)C in a humidified atmosphere of 5% CO\(_2\) in air for at least 4 days. Colony counts were performed from dilutions yielding between 30 and 300 colonies per plate and values were converted to c.f.u. ml\(^{-1}\), taking the dilution of mycoplasma that occurred during experiments into consideration. The limit of detection was 10\(^5\) c.f.u. ml\(^{-1}\) and experiments were performed on two occasions to ensure reproducibility.

PD analysis. Antimycoplasmal effect, defined as the change in log\(_{10}\) (c.f.u. ml\(^{-1}\)) over a 12 h period, and the 12 h-area under curve (AUC\(_{12\ h}\)) were calculated for each concentration of danofloxacin (single-release bolus only) and tulathromycin (single-release bolus and infusion). Phoenix WinNonlin 6.2 professional software (Pharsight Corporation) was used to fit data to a sigmoidal E\(_{\text{max}}\) model given by the formula \(E=E_0+(E_{\text{max}}-E_0)C^N/(EC_{50}^N+C^N)\), where \(E\) is the antimycoplasmal effect, \(E_0\) is the difference in log\(_{10}\) (c.f.u. ml\(^{-1}\)) after 12 h compared with the initial titre when no antimicrobial is present, \(E_{\text{max}}\) is the maximum antimycoplasmal effect, EC\(_{50}\) is the AUC\(_{12\ h}\) that gives rise to 50% of the maximum response, \(C\) is the AUC\(_{12\ h}\) ratio in the effect compartment (i.e. artificial medium) and \(N\) is the Hill coefficient, which reflects the slope of the relationship between antimycoplasmal effect and AUC\(_{12\ h}\). Values for \(E_{\text{max}}\) between 0 and \(-3\) log\(_{10}\) (c.f.u. ml\(^{-1}\)) indicated mycoplasmastasis, whereas values less than or equal to \(-3\) log\(_{10}\) (c.f.u. ml\(^{-1}\)) denoted mycoplasmacidal activity.

RESULTS AND DISCUSSION

MICs for OTC, danofloxacin and tulathromycin against MmmsSC strain B237 were determined previously at an inoculum size of 10\(^7\) c.f.u. ml\(^{-1}\). This was the intended initial titre for subsequent time–kill assays, which had to be high to ensure that the maximum effects of antimicrobials were captured. In addition, high inoculum sizes may reflect mycoplasmal density at the infection site more closely (Levison, 2004) and are more likely to contain subpopulations of mycoplasma that are less susceptible to drugs, if any, as well as the fully susceptible subpopulation (Kestem et al., 2009). Therefore, defining dosage protocols using high inoculum sizes is less likely to result in the selection of drug-resistant organisms.

Representative time–kill curves for OTC, danofloxacin and tulathromycin activity against MmmsSC strain B237 are shown in Fig. 1, with sigmoidal E\(_{\text{max}}\) models based on mean data for the latter two antimicrobials in Fig. 2. Over 12 h, OTC was mycoplasmastatic at all concentrations tested, regardless of whether administration was by single-release bolus or infusion. In particular, no mycoplasmal growth was observed at a concentration of 1 \(\times\) MIC when administered as a single-release bolus. However, this was not unexpected as, given the long half-life of OTC (Craigmill et al., 2004), the concentration at 12 h would have been \(-0.27\) mg l\(^{-1}\). Indeed, growth of only 0.27 and 0.15 log\(_{10}\) (c.f.u. ml\(^{-1}\)) was observed when MmmsSC was exposed to fixed concentrations of 0.25 and 0.30 mg l\(^{-1}\), respectively, for 24 h (data not shown). Furthermore, a concentration of 1 \(\times\) MIC may induce a post-antibiotic effect, which, when coupled with subMIC concentrations, may have resulted in growth inhibition.
Of the concentrations tested, danofloxacin caused the greatest kill when administered as a single-release bolus of 16 × MIC. In terms of the amount of drug, this is approximately equivalent to an infusion of 2 × MIC. Whilst the former elicited a mean kill of 1.65 log₁₀ (c.f.u. ml⁻¹) over 12 h, the latter resulted in a kill of only 1.02
log_{10} (c.f.u. ml^{-1}). Similarly, a single-release bolus of 8 \times \text{MIC} is approximately equivalent to an infusion of 1 \times \text{MIC}. Again, greater kill was achieved with single-release bolus administration [1.32 vs 0.27 log_{10} (c.f.u. ml^{-1})]. This suggested that danofloxacin possesses mainly concentration-dependent activity against \textit{MmmSC}. However, mycoplasmacidal activity, defined as a reduction in the initial inoculum size by 3 log_{10} (c.f.u. ml^{-1}), was not observed for danofloxacin over 12 h, as the maximum antimycoplasmal effect (\(E_{\text{max}}\)) was only \(-1.91\) log_{10} (c.f.u. ml^{-1}) (Table 1). In a fixed-concentration model, danofloxacin elicited mycoplasmacidal activity over 24 h (Mitchell et al., 2011). Development of a continuous-dilution model will enable longer experiments and assessment of any post-antibiotic effects (Gloede et al., 2010; Meletiadis et al., 2012). However, as a result of its relatively short half-life, using dynamic models to assess danofloxacin activity against \textit{MmmSC} suffers from the problem that dilution or flow rates are almost equivalent to the mycoplasmal growth rate. Only 0.15 log_{10} (c.f.u. ml^{-1}) of growth was observed in the control (0 \times \text{MIC}) in the current study. Therefore, interpretation as to whether mycoplasmastasis affected by single-release bolus administration of 1 \times \text{MIC} was a result of post-antibiotic effects or lack of assay sensitivity to detect significant growth at this concentration was not possible.

Tulathromycin produced a similar activity against \textit{MmmSC} whether it was administered as a single-release bolus or infusion. Indeed, comparable values were obtained for the parameters that describe the sigmoidal \(E_{\text{max}}\) models (Table 1). However, this was not unexpected owing to the long plasma half-life (65 h) of this drug (Nowakowski et al., 2004). As for danofloxacin, mycoplasmacidal activity was not attained over 12 h [\(E_{\text{max}}\) as log_{10} (c.f.u. ml^{-1})]: single-release bolus, \(-0.93\); infusion, \(-1.01\)] and therefore a continuous flow model is required to determine activity at further time points. In addition, growth akin to the control (0 \times \text{MIC}) was observed for single-release bolus administration of 1 \times \text{MIC} and infusions of 1 and 1.5 \times \text{MIC}. Again, this was not completely unexpected, as \textit{MmmSC} in the presence of tulathromycin at 1 \times \text{MIC} grew at a similar rate to that of the control over at least the first 8 h in fixed-concentration models, with mycoplasmacidal activity not

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<tr>
<th>Variable</th>
<th>Danofloxacin</th>
<th>Tulathromycin</th>
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<tr>
<td></td>
<td>Single-release bolus</td>
<td>Single-release bolus</td>
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<td>(E_0)</td>
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<td>(N)</td>
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<td>3.63</td>
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Table 1. PD analysis for danofloxacin (single-release bolus only) and tulathromycin (single-release bolus and infusion) activity against \textit{MmmSC} strain B237.

The relationships between AUC_{12 h}:MIC ratio and antimycoplasmal effect were described using sigmoidal \(E_{\text{max}}\) models, which are parameterized by the following: \(E_0\) is the difference in log_{10} (c.f.u. ml^{-1}) after 12 h compared with the initial titre when no antimicrobial is present; \(E_{\text{max}}\) is the maximum antimycoplasmal effect; EC_{50} is the AUC_{12 h}:MIC ratio that gives rise to 50% of the maximum response; and \(N\) is the Hill coefficient, which reflects the steepness of the slope.

Fig. 2. Sigmoidal \(E_{\text{max}}\) relationships for AUC_{12 h}:MIC ratio versus antimycoplasmal effect for danofloxacin single-release bolus (a), tulathromycin single-release bolus (b) and tulathromycin infusion (c) in a dynamic stepwise dilution model. Data points represent means of two experiments.
commencing until sometime between 8 and 24 h (data not shown).

The main purpose of this study was to compare the efficacy of OTC, danofloxacin and tulathromycin against MmmSC using an in vitro dynamic concentration model, and therefore the mean values for the half-lives of these drugs were selected. However, we recognize that there is inter-individual variability for this parameter within a population. For example, Giles et al. (1991) report elimination rate constants of 0.124–0.247 h\(^{-1}\) for danofloxacin, yielding half-lives of 2.81–5.59 h. The PK/PD parameters that are most predictive of fluoroquinolone efficacy are AUC:MIC and maximum plasma concentration (C\(_{\text{max}}\)) : MIC ratios (Lees et al., 2006). For a given C\(_{\text{max}}\) value (initial concentration in this model), changing the half-life would result in a different AUC:MIC ratio, and variation in maximum antimycoplasmal effects may be observed. Therefore, experiments performed at a range of half-lives reflecting the population data should be considered for future studies.

The results of this study showed that all three antimicrobials were effective against MmmSC strain B237 in an in vitro dynamic PK/PD model. Although OTC caused no decrease in titre from initial inoculum size at the concentrations tested, it is important to understand that these studies do not consider the host’s immune response, which may act additively or synergistically with antimicrobial activity (Lees et al., 2006; Levison, 2004). In addition, OTC, as well as tilmicosin, has been shown to have the potential for anti-inflammatory effects (Fischer et al., 2011; Rempe et al., 2007), which may reduce the pathology associated with CBPP. Therefore, OTC remains a potential candidate for control of this disease. In contrast, danofloxacin and tilmicosin resulted in a decrease in mycoplasmal titre, although they did not achieve mycoplasmacidal activity over 12 h. Whilst the method of drug exposure was irrelevant for tilmicosin activity, owing to its long half-life, the data suggested that danofloxacin possesses concentration-dependent activity. This indicates that a better outcome is likely to be achieved by giving the total dose of danofloxacin as a single-release bolus rather than giving smaller divided doses (Levison, 2004).

**ACKNOWLEDGEMENTS**

We thank Joachim Frey at the University of Bern for supplying MmmSC strain B237 and Pfizer for donating tilmicosin. This work was supported by the Biotechnology and Biological Sciences Research Council (grant reference: BB/H00945/01).

**REFERENCES**


