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 (*MmmSC*) 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 *MmmSC 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 MmmsC. Fixed-concentration models have been used to investigate the activity of OTC, danofloxacin and tulathromycin against MmmsC 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 MmmsC 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.** MmmsC 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 in powder form 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 °C until use. Fresh solutions of OTC hydrochloride were prepared in ddH₂O 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 MmmsC.

The MICs for OTC, danofloxacin and tulathromycin against MmmsC 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 10⁷ c.f.u. ml⁻¹ (Mitchell et al., 2011).

**Time–kill assays.** To prepare the inoculum, mycoplasma were cultured in pre-warmed liquid medium (Mycoplasma Experience) at 37 °C and diluted while in the exponential phase to give an inoculum of 10⁷ 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 × MIC of OTC and danofloxacin, 1–64 × MIC of tulathromycin) or infusion (1, 1.5 and 2 × MIC of all three antimicrobials, plus 3, 4, 7 and 14 × MIC for tulathromycin). Growth controls were included (0 × MIC) and cultures were incubated at 37 °C. To mimic elimination of drugs from the body, appropriate amounts of drug-free (single-release bolus) or drug-containing (infusion) liquid medium were added at 30 min intervals to reduce the antimicrobial concentration according to the equation C = C₀e⁻kt, where C is antimicrobial concentration at time t, C₀ is the initial antimicrobial concentration and k is the elimination rate constant. Elimination rate constants were calculated using k = ln2/τmax, where half-life (τmax) was taken from the literature [21.6 h for long-acting OTC (Craigmill et al., 2004), 4.01 h for danofloxacin (Giles et al., 1991) and 65 h for tulathromycin (Nowakowski et al., 2004)]. Such methodology has been used previously to investigate the activity of moxifloxacin against Bacteroides fragilis and Escherichia coli (Schaumann et al., 2005).

Experiments were conducted over a 12 h period. Samples (10 µl) were taken at 0 h and every 2 h thereafter and serially diluted tenfold down to 10⁻⁴. Aliquots (10 µl) of each dilution were transferred to solid medium (Mycoplasma Experience) and incubated at 37 °C in a humidified atmosphere of 5% CO₂ 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 10⁵ c.f.u. ml⁻¹ and experiments were performed on two occasions to ensure reproducibility.

**PD analysis.** Antimycoplasmal effect, defined as the change in log₁₀ (c.f.u. ml⁻¹) over a 12 h period, and the 12 h-area under curve (AUC₁₂):MIC ratio 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 sigmoid Eₘₐₓ model given by the formula E = E₀ + ((Eₘₐₓ − E₀)C₀N)/(EC₅₀N + C₀N), where E is the antimycoplasmal effect, E₀ is the difference in log₁₀ (c.f.u. ml⁻¹) after 12 h compared with the initial titre when no antimicrobial is present, Eₘₐₓ is the maximum antimycoplasmal effect, EC₅₀ is the AUC₁₂:MIC ratio that gives rise to 50% of the maximum response, C₀ is the AUC₁₂:MIC 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 AUC₁₂:MIC ratio. Values for Eₘₐₓ between 0 and −3 log₁₀ (c.f.u. ml⁻¹) indicated mycoplasmastasis, whereas values less than or equal to −3 log₁₀ (c.f.u. ml⁻¹) denoted mycoplasmacidal activity.

**RESULTS AND DISCUSSION**

MICs for OTC, danofloxacin and tulathromycin against MmmsC strain B237 were determined previously at an inoculum size of 10⁷ 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 MmmsC strain B237 are shown in Fig. 1, with sigmoidal Eₘₐₓ 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 × 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 log₁₀ (c.f.u. ml⁻¹) was observed when MmmsC 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 × 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 $16 \times \text{MIC}$. In terms of the amount of drug, this is approximately equivalent to an infusion of $2 \times \text{MIC}$. Whilst the former elicited a mean kill of $1.65 \log_{10} \text{(c.f.u. ml}^{-1})$ over 12 h, the latter resulted in a kill of only $1.02 \log_{10} \text{(c.f.u. ml}^{-1})$. 

**Fig. 1.** Representative time–kill curves for OTC (a), danofloxacin (b), tulathromycin (single-release bolus) (c) and tulathromycin (infusion) (d) against MmmSC strain B237 in a dynamic stepwise dilution model.
log_{10} (c.f.u. ml^{-1}). Similarly, a single-release bolus of 8 x MIC is approximately equivalent to an infusion of 1 x 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 MmmSC. However, mycoplasmal 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_{max}) was only −1.91 log_{10} (c.f.u. ml^{-1}) (Table 1).

In a fixed-concentration model, danofloxacin elicited mycoplasmal 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 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 x MIC) in the current study. Therefore, interpretation as to whether mycoplasmastasis affected by single-release bolus administration of 1 x 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 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_{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, mycoplasmal activity was not attained over 12 h [E_{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 x MIC) was observed for single-release bolus administration of 1 x MIC and infusions of 1 and 1.5 x MIC. Again, this was not completely unexpected, as MmmSC in the presence of tulathromycin at 1 x MIC grew at a similar rate to that of the control over at least the first 8 h in fixed-concentration models, with mycoplasmal activity not

Table 1. PD analysis for danofloxacin (single-release bolus only) and tulathromycin (single-release bolus and infusion) activity against MmmSC strain B237

The relationships between AUC_{12h}: MIC ratio and antimycoplasmal effect were described using sigmoidal E_{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_{max} is the maximum antimycoplasmal effect; EC_{50} is the AUC_{12h}:MIC ratio that gives rise to 50 % of the maximum response; and N is the Hill coefficient, which reflects the steepness of the slope.
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 interindividual 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_max) : MIC ratios (Lees et al., 2006). For a given C_max value (initial concentration in this model), changing the half-life would result in a different AUC : MIC ratio, and variation in maximum antibioticplasmal 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 tulathromycin, 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 tulathromycin 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 tulathromycin 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 tulathromycin. This work was supported by the Biotechnology and Biological Sciences Research Council (grant reference: BB/H00945/01).

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