Pharmacokinetic/pharmacodynamic integration and modelling of oxytetracycline for the porcine pneumonia pathogens Actinobacillus pleuropneumoniae and Pasteurella multocida

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INTRODUCTION

The tetracycline group of antimicrobial drugs, discovered in 1948, has consistently had the highest veterinary sales volume of the seven drug classes analysed by UK-VARSS (2014). Resistance to oxytetracycline may be similar to that reported for tetracycline, but this remains to be confirmed. Nevertheless, according to the Clinical Laboratory Standards Institute (CLSI, 2013), whilst tetracycline is the class representative, MIC and breakpoint interpretation for tetracycline also apply to oxytetracycline.

Oxytetracycline is a broad-spectrum drug, classified as a bacteriostat, when administered at clinical dosages, but in vitro studies have demonstrated bactericidal actions (Brentnall et al., 2012; Dorey et al., 2016; Lees et al., 2016). Several pathogens implicated in pig pneumonia, including mycoplasma species, were shown historically to be susceptible to the actions of oxytetracycline. It has been registered for veterinary use in most European countries and has been in extensive use in farm animals for more than 60 years. It is therefore still a drug of considerable scientific and clinical interest.

For the treatment of porcine pneumonia, oxytetracycline has the advantage of low cost, clinical efficacy in at least some cases and availability in a long-acting formulation (Lees & Toutain, 2011). Licensed 20–30% w/v strength parenteral formulations of oxytetracycline have persistent actions, because of the high strength and high dosage used (20–30 mg/kg), leading to sustained absorption from the reservoir site of intramuscular injection. This gives rise to flip-flop pharmacokinetics (PK) (Nouws & Vree, 1983; Toutain & Raynaud, 1983; Nouws et al., 1990). In recent years, there have been major advances in designing dosage schedules of antimicrobial drugs, based on integration and modelling of pharmacodynamic (PD) and PK data. These approaches have provided novel strategies for predicting drug dosages that optimize efficacy and minimize opportunities for the emergence of resistance (Nielsen et al.,...
Extensively to generate three PK/PD indices, namely the ratios, potency indices with et al. (Blondeau et al., 2004). Integration of in vitro generated potency indices with in vivo-generated PK data has been used extensively to generate three PK/PD indices, namely the ratios, maximum plasma concentration (C_{max})/MIC and area under plasma concentration–time curve (AUC_{24 h})/MIC, and time (T) > MIC, the time for which concentration exceeds MIC. Integrated PK/PD data are commonly supplemented by in vitro time–kill studies. These generate information on the time course of antimicrobial action, enabling classification of drugs as time-, concentration- or co-dependent in their killing actions. From time–kill studies, numerical values of PK/PD breakpoints can be determined by PK/PD modelling (Aliabadi & Lees, 2002; Toutain & Lees, 2004; Mouton et al., 2011; Martinez et al., 2012; Nielsen & Friberg, 2013; Papich, 2014; Sidhu et al., 2014; Potter et al., 2013). These PK and PD data may be used additionally, with wild-type MIC distributions of susceptible pathogens, to conduct Monte Carlo simulations (MCS) to predict doses providing a range of predetermined levels of kill. Ideally, predicted doses are then correlated with clinical and bacteriological cures in animal disease models and clinical trials (Nielsen et al., 2011; Nielsen & Friberg, 2013; Papich, 2014; Sidhu et al., 2014; Lees et al., 2015b). With the aims of (i) extending the useful life of older antimicrobial drugs and (ii) reducing the likelihood of resistance emergence and ensuring prudent use of these drugs, there have been proposals to re-evaluate dose schedules that were set, in many instances, many years ago (Aliabadi & Lees, 2001; Mouton et al., 2011, 2012; Nielsen et al., 2011; Martinez et al., 2012; Nielsen & Friberg, 2013; Nyberg et al., 2014; Papich, 2014; Rey et al., 2014; Toutain et al., 2017). The European Union Committee on Antimicrobial Testing (EUCAST) approach to dosage re-evaluation has been described by Mouton et al. (2011). Moreover, the above-named authors have recognized that a sound scientific approach to setting dose schedules of antimicrobial drugs is to link PK parameters and variables with appropriate indices of potency, applying the general equation for systemically acting drugs (Fig. 1) (Aliabadi & Lees, 2001, 2002; Toutain & Bousquet-Melou, 2004; Toutain & Lees, 2004).

The internationally accepted EUCAST and CLSI methods and standards for MIC determinations are based on the use of artificial broths. These are nonbiological growth media, such as Mueller Hinton Broth (MHB), formulated on a pathogen-by-pathogen basis. Whilst such media are specifically designed to provide optimal in vitro growth conditions, they differ in composition from body fluids. Clinical treatment of disease depends on drug concentration in the biological fluid of the biophase. Concentration in the latter is driven by the plasma concentration of free drug. Therefore, for some drug classes, including tetracyclines and triamilides, it has been proposed that the use of serum to determine MIC might be more relevant than reliance on determinations in broths (Nightingale & Murakawa, 2002; Brentnall et al., 2013; Honeyman et al., 2015; Lees et al., 2016; Toutain et al., 2017). This is justified by the fact that serum, whilst not identical to biophase fluids, such as pulmonary epithelial lining fluid, is for a given species much closer in composition than artificial broths.

For the same reason, namely closer approximation to bacterial growth conditions in vivo, comparative time–kill studies conducted in our laboratory, based on multiples of MIC, have been conducted in both broths and serum of the species of interest to establish matrix differences, if any (Illlambas et al., 2013a,b; Potter et al., 2013; Sidhu et al., 2014; Lees et al., 2015a,b). In vitro time–kill data allow classification of killing action as time-, concentration- or co-dependent and, by modelling the data, PK/PD breakpoints have been generated for a given drug against a given pathogen. In conjunction with PK data and MIC distributions of wild-type organisms, PK/PD

\[
DO = \frac{CI \times \text{Breakpoint Index} \times \text{Targeted plasma concentration}}{F \%} \times MIC_{50} \quad (\mu g/mL)
\]

DO: Daily dose (mg/kg/day)
CI: Body Clearance (per h)
Breakpoint index: Index for required effect (AUC_{24 h}/MIC) (h)
Targeted plasma concentration: MIC_{50} (\mu g/mL)
F%: Bioavailability (from 0 to 1)

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Fig. 1. Formula for calculation of daily drug dose based on pharmacokinetic and pharmacodynamic variables.
breakpoints have been used to predict dosages for a range of target attainment rates (TAR) (Toutain & Lees, 2004; Sidhu et al., 2010, 2014; Toutain et al., 2017).

The aims of this study were (i) to determine the plasma concentration–time profile for oxytetracycline administered to pigs intramuscularly at the recommended dosage of 20 mg/kg and derive PK variables by noncompartmental analysis; (ii) to integrate in vivo PK variables with in vitro PD indices of potency (MIC and MPC) to determine values of $C_{\text{max}}$/MIC, $C_{\text{av}}$/MIC, $T > MIC$, $T > MPC$ and ratios of average concentration ($C_{\text{av}}$/MIC and $C_{\text{av}}$/MPC for $A.\ pleuropneumoniae$ and Pasteurella multocida; (iii) to model data from time–kill studies of $A.\ pleuropneumoniae$ and $P.\ multocida$ in order to generate PK/PD breakpoint values of $AUC_{24\ h}$/MIC for three levels of bacterial kill, bacteriostasis, bactericidal and 4 log$_{10}$ reduction in inoculum count; (iv) to use PK and PK/PD breakpoints, with serum protein binding data and literature MIC distributions in Monte Carlo simulations to estimate dose schedules required for: (i) bacteriostatic and bactericidal levels of kill; (ii) for 50% and 90% TAR; and (iii) for single dosing and daily dosing at steady-state.

MATERIALS AND METHODS

Origin, storage and selection of bacterial isolates
Twenty isolates of $P.\ multocida$ were supplied by Don Whitley Scientific (Shipley, West Yorkshire, UK). They also supplied three ATCC reference strains, $A.\ pleuropneumoniae$ ATCC 27090, Enterococcus faecalis ATCC 29212 and Escherichia coli ATCC 25922, to validate MIC determinations. Eight isolates of $A.\ pleuropneumoniae$ were supplied by A. Rycroft (Royal Veterinary College, Hawkshead Campus, Hatfield, Herts., UK). All $P.\ multocida$ and $A.\ pleuropneumoniae$ isolates were derived from EU field cases of pig pneumonia. Based on three criteria, six isolates of each species were selected: (i) ability to grow logarithmically in both broth and pig serum; (ii) susceptibility to oxytetracycline in disc diffusion assays (data not shown); and (iii) the highest and lowest broth MICs and four isolates with intermediate MICs, determined using twofold dilutions (data not shown). This initial selection procedure ensured that all isolates could be used in subsequent investigations in both growth media and that they comprised a small but diverse range of susceptible isolates.

Determination of minimum inhibitory and mutant prevention concentrations
Minimum inhibitory concentrations were determined by microdilution for six isolates each of $A.\ pleuropneumoniae$ and $P.\ multocida$, except where stated in accordance with CLSI guidelines (2013), using artificial broths, cation-adjusted Mueller Hinton Broth (CAMHB) for $P.\ multocida$ and Columbia Broth (CB) supplemented with nicotinamide adenine dinucleotide (NAD) for $A.\ pleuropneumoniae$. CLSI recommended artificial medium for $A.\ pleuropneumoniae$ culture is veterinary fastidious medium; this was replaced for CB as improved bacterial growth was determined and the lack of blood clot formation made MIC endpoints easier to establish. To improve accuracy of estimates for each isolate, five sets of overlapping twofold serial dilutions of oxytetracycline were prepared in 96-well plates and each determination was made in triplicate, instead of the CLSI standard which uses single twofold dilutions. This was considered necessary, because of the small number of isolates of each species used in this study. In addition, the guidelines were adapted using pig serum in place of broth to enable comparison of potency in the two matrices, again with five overlapping sets of twofold dilutions and determinations in triplicate. Methods were as described by Dorey et al. (2016).

Mutant prevention concentrations were determined by applying a high count bacterial suspension ($1–2 \times 10^{11}$ colony-forming units (CFU)/mL) onto an agar plate containing drug concentrations 1, 2, 4, 8, 16, 32, 64 and 128 multiples of the MIC for each isolate. The concentration ranges were narrowed down two further times. Plates were incubated at 37 °C for 72 h and checked for growth every 24 h. MPC was the lowest oxytetracycline concentration inhibiting bacterial growth completely after 72-h incubation. The method was validated against that developed by Blondeau et al. (2004) and described by Dorey et al. (2016). Each experiment was repeated in triplicate for six isolates of each test organism in broth and serum matrices.

Oxytetracycline pharmacokinetics
Individual animal plasma concentration–time profiles for oxytetracycline were supplied by Norbrook Laboratories Ltd. for two oxytetracycline containing products (Alamycin LA, Norbrook Laboratories, Northern Ireland and Terramycin LA, Pfizer, UK). The study was approved by the company’s Ethics Committee and was Good Laboratory Practice compliant. All pigs were Landrace Cross males, aged 2 months. Each product was administered intramuscularly at a dosage of 20 mg/kg. As the two products had been shown to be bioequivalent, the data were pooled, to provide a data set of 14 animals ($n = 6$ for Terramycin LA and $n = 8$ for Alamycin LA) for noncompartmental analysis (NCA) using WinNonLin V6.5 (Pharsight Corporation, Mountain View, CA, USA). Variables calculated were $C_{\text{max}}$, $C_{\text{av}}$, $AUC$, area under first moment curve ($AUMC$), time of maximum concentration ($T_{\text{max}}$), clearance scaled by bioavailability (CL/F), terminal half-life ($T_{1/2}$) and mean residence time from the time of dosing to the time of last measurable concentration ($\text{MRT}_{\text{last}}$). Three time periods were investigated (0–48 h, 0–24 h and 24–48 h) for determination of average concentrations.

PK/PD integration
Pharmacokinetic–pharmacodynamic indices, calculated using individual animal PK values and mean MIC and MPC values, were as follows: (i) $C_{\text{max}}$/MIC, $T > MIC$ and $C_{\text{av}}$/MIC ratios for
For each pathogen, in vitro growth inhibition curves for oxytetracycline were determined using eight multiples of MIC, as previously described (Dorey et al., 2016). Each test was repeated in triplicate for six isolates each of *A. pleuropneumoniae* and *P. multocida* in both broth (CB and CAMHB, respectively) and pig serum. The lower limit of quantification (LLOQ) was 33 CFU/mL. The sigmoidal E\(_{\text{max}}\) equation (Fig. 2) was then used to model AUC\(_{24\ h}\)/MIC data. Using the calculated parameters with this equation, log\(_{10}\) change in CFU/mL against AUC\(_{24\ h}\)/MIC was simulated (Fig. 3). AUC\(_{24\ h}/\text{MIC}\) PK/PD breakpoints were determined for three levels of growth inhibition: \(E = 0\), bacteriostatic, that is 0 log\(_{10}\) reduction in CFU/mL after 24-h incubation; \(E = -3\), bactericidal, 3 log\(_{10}\) reduction in CFU/mL; and \(E = -4\), 4 log\(_{10}\) reduction in bacterial count.

**Dose determination**

For dose prediction using the deterministic approach, average values of PK and PK/PD breakpoint values were used, together with MIC\(_{90}\) values for *P. multocida* and *A. pleuropneumoniae* for tetracycline obtained from the literature (de Jong et al., 2014).

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**Fig. 2.** The sigmoidal E\(_{\text{max}}\) equation used to model time–kill data by nonlinear regression (Lees et al., 2015a).

**Fig. 3.** Typical example plot of AUC\(_{24\ h}/\text{MIC}\) vs. change in bacterial count (log\(_{10}\) CFU/mL) obtained from in vitro time–kill data for oxytetracycline. Each point represents an experimental value showing time–kill 24-h data point of one isolate, one repeat and one matrix. The curve is the line of best fit based on the sigmoidal E\(_{\text{max}}\) equation. [Colour figure can be viewed at wileyonlinelibrary.com].

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MIC distribution data for oxytetracycline were not available from a European source (vide infra). However, MIC and breakpoint interpretation for tetracycline also apply to oxytetracycline, according to CLSI (CLSI, 2013).

For dose determination using Monte Carlo simulations, data input comprised: (i) whole body clearance scaled by bioavailability; (ii) free drug concentration in plasma; (iii) $AUC_{24\ h}$/MIC breakpoints derived from time–kill curves by PK/PD modelling; and (iv) MIC field distribution data for tetracycline as indicated above (Fig. 4). In addition, for comparative purposes, more limited Japanese oxytetracycline MIC distribution data for *P. multocida* in pigs were used (Yoshimura *et al.*, 2001). Monte Carlo simulations were based on numerical values and incidence of each input variable and predicted: the daily dose at steady-state (Fig 5); single loading doses for three time periods, 0–24, 0–48 and 0–72 h; and doses for three levels of bacterial kill, as described by Lees *et al.* (2015a). All dosages were computed using Monte Carlo simulations in Oracle Crystal Ball (Oracle Corporation, Redwood Shores, CA, USA) for TAR of 50% and 90%. For the literature MIC distributions, values were corrected using the serum:broth MIC ratio, as the reported MIC literature values were determined in broth. The probabilities of distribution for the dosage estimation were run for 50 000 simulated trials.

**RESULTS**

**Minimum inhibitory and mutant prevention concentrations**

Minimum inhibitory concentrations and MPCs were determined for six isolates each of *P. multocida* and *A. pleuropneumoniae*, and each was determined in broth and pig serum. Geometric mean MIC values in $\mu$g/mL (SD) for *P. multocida* were 0.30 (0.12) and 6.48 (1.45), respectively. Corresponding values for *A. pleuropneumoniae* were 2.50 (0.70) and 2.08 (0.43) (Dorey *et al.*, 2016). Mean MPC values in $\mu$g/mL (SD) for *P. multocida* were 6.84 (2.76) and 142.8 (33.3) determined in broth and serum, respectively. For *A. pleuropneumoniae*, MPC values were 44.4 (12.63) and 35.1 (6.66) $\mu$g/mL, respectively (Dorey *et al.*, 2016). For each pathogen, MPC:MIC ratios were similar in serum and broth.

**Oxytetracycline pharmacokinetics**

From plasma concentration–time data, PK variables were calculated for 14 pigs, comprising data for six animals receiving product A (Terramycin LA) and eight pigs administered product B (Alamycin LA). Each product was administered intramuscularly at a dosage of 20 mg/kg, and the two products

![Fig. 4. Tetracycline MIC frequency distributions for *P. multocida* (n = 105) and *A. pleuropneumoniae* (n = 110). Data obtained using CLSI methods (de Jong *et al.*, 2014). Sampling period covered 2002–2006 from European countries. Oxytetracycline MIC frequency distribution for *P. multocida* (n = 61). MIC data obtained using agar dilution method. Sampling period covered 1994–1998 from Japan. [Colour figure can be viewed at wileyonlinelibrary.com].](image-url)
were bioequivalent. Table 1 indicates mean values for each variable, determined by noncompartmental analysis.

**PK/PD integration**

Pharmacokinetic–pharmacodynamic integration established the parameters \( C_{\text{max}}/\text{MIC} \), \( T > \text{MIC} \) and concentration averages \( (C_{\text{av}})/\text{MIC} \), over three time periods (0–48, 0–24 and 24–48 h) (Table 2). Integrated values were numerically much greater for broth MIC than for serum MIC for \( P. \) multocida; \( C_{\text{av0–24}}/\text{MIC} \) was 9.01 in broth and 0.42 in serum, but for \( A. \) pleuropneumoniae data were similar between the two media, \( C_{\text{av0–24}}/\text{MIC} \) being 1.08 and 1.30, respectively. For \( P. \) multocida, the plasma concentration remained above the broth MIC for 52.5 h, and \( T > \text{MIC} \) for serum was 3.62 h. Corresponding values for \( A. \) pleuropneumoniae were 11.0 and 15.5 h.

Integration of PK and PD data for MPC is presented in Table 3. All ratios were much lower than the PK:\PD ratios, as a consequence of the MPC:MIC ratios, which were 22.8:1 (serum) and 22.0:1 (broth) for \( P. \) multocida and 16.9:1 (serum) and 17.9:1 (broth) for \( A. \) pleuropneumoniae. \( C_{\text{max}}/\text{MPC} \) ratios were less than 1 for both pathogens and both matrices.

**Table 1.** Pharmacokinetic variables (mean, standard deviation, \( n = 14 \)) for oxytetracycline

| Variable       | Units | Mean | SD   |
|----------------|-------|------|------|
| \( C_{\text{max}} \) | mg/L  | 5.67 | 2.40 |
| \( AUC \)       | h-mg/L| 84.5 | 14.7 |
| \( AUMC \)      | h-mg/L| 1244 | 221  |
| \( T_{\text{max}} \) | h     | 0.92 | 1.05 |
| \( \text{CI/F} \) | L/h/kg| 0.22 | 0.04 |
| \( T_{1/2} \)    | h     | 12.9 | 1.83 |
| \( \text{MRT}_{\text{last}} \) | h     | 14.7 | 1.27 |

Pharmacokinetic variables were determined by noncompartmental analysis for oxytetracycline administered intramuscularly at a dosage of 20 mg/kg. Values are geometric means except for \( T_{\text{max}} \) (arithmetic mean) and \( T_{1/2} \) (harmonic mean). \( C_{\text{max}} \), maximum concentration; \( AUC \), area under plasma concentration–time curve; \( AUMC \), area under first moment curve; \( T_{\text{max}} \), time taken to reach maximum concentration; \( \text{CI/F} \), drug clearance scaled by bioavailability; \( T_{1/2} \), terminal half-life; \( \text{MRT}_{\text{last}} \), mean residence time from the time of dosing to the time of last measurable concentration.

**Table 2.** Integration of pharmacokinetic and pharmacodynamic variables for oxytetracycline for broth and serum MICs (mean and standard deviation)

| Organism      | Parameter       | Broth | Serum |
|---------------|-----------------|-------|-------|
|               | Units           | Mean  | SD    | Mean  | SD    |
| \( Pasteurella \) & \( multocida \) & |       |       |       |       |
| \( C_{\text{av0–48}}/\text{MIC} \) | \( C_{\text{av0–24}}/\text{MIC} \) & 5.87 | 6.02 | 0.27 | 0.05 |
| \( C_{\text{av0–24}}/\text{MIC} \) | \( C_{\text{av24–48}}/\text{MIC} \) & 9.01 | 1.75 | 0.42 | 0.08 |
| \( C_{\text{av24–48}}/\text{MIC} \) | \( C_{\text{av0–24}}/\text{MIC} \) & 3.28 | 0.81 | 0.15 | 0.04 |
| \( C_{\text{max}}/\text{MIC} \)       | \( C_{\text{max}}/\text{MIC} \) & 18.9 | 7.99 | 0.88 | 0.37 |
| \( T > \text{MIC} \)                | \( h \)       & 52.5 | 5.70 | 3.62 | 7.13 |
| \( Actinobacillus \) & \( pleuropneumoniae \) & |       |       |       |       |
| \( C_{\text{av0–48}}/\text{MIC} \) | \( C_{\text{av0–24}}/\text{MIC} \) & 0.70 | 0.12 | 0.85 | 0.15 |
| \( C_{\text{av0–24}}/\text{MIC} \) | \( C_{\text{av24–48}}/\text{MIC} \) & 1.08 | 0.21 | 1.30 | 0.25 |
| \( C_{\text{av24–48}}/\text{MIC} \) | \( C_{\text{av0–24}}/\text{MIC} \) & 0.39 | 0.10 | 0.47 | 0.12 |
| \( C_{\text{max}}/\text{MIC} \)       | \( C_{\text{max}}/\text{MIC} \) & 2.27 | 0.96 | 2.73 | 1.15 |
| \( T > \text{MIC} \)                | \( h \)       & 11.0 | 3.71 | 15.5 | 3.38 |

Individual animal pharmacokinetic data for 14 animals divided by mean MICs for six isolates of each species measured in broth and serum. \( C_{\text{av}} \), average concentration calculated for time periods 0–48, 0–24 and 24–48 h; \( C_{\text{max}} \), maximum plasma concentration (\( \mu g/mL \)); \( T > \text{MIC} \), time for which plasma concentration exceeds MIC (h).

**Table 3.** Integration of pharmacokinetic and pharmacodynamic variables for oxytetracycline for broth and serum MPCs (mean and standard deviation)

| Organism      | Parameter       | Broth | Serum |
|---------------|-----------------|-------|-------|
|               | Units           | Mean  | SD    | Mean  | SD    |
| \( Pasteurella \) & \( multocida \) & |       |       |       |       |
| \( C_{\text{av0–48}}/\text{MPC} \) | \( C_{\text{av0–24}}/\text{MPC} \) & 0.26 | 0.04 | 0.01 | 0.00 |
| \( C_{\text{av0–24}}/\text{MPC} \) | \( C_{\text{av24–48}}/\text{MPC} \) & 0.40 | 0.08 | 0.02 | 0.00 |
| \( C_{\text{av24–48}}/\text{MPC} \) | \( C_{\text{av0–24}}/\text{MPC} \) & 0.14 | 0.04 | 0.01 | 0.00 |
| \( C_{\text{max}}/\text{MPC} \)       | \( C_{\text{max}}/\text{MPC} \) & 0.83 | 0.35 | 0.04 | 0.02 |
| \( T > \text{MPC} \)                | \( h \)       & 0     | 0     | 0     | 0     |
| \( Actinobacillus \) & \( pleuropneumoniae \) & |       |       |       |       |
| \( C_{\text{av0–48}}/\text{MPC} \) | \( C_{\text{av0–24}}/\text{MPC} \) & 0.04 | 0.01 | 0.05 | 0.01 |
| \( C_{\text{av0–24}}/\text{MPC} \) | \( C_{\text{av24–48}}/\text{MPC} \) & 0.06 | 0.01 | 0.08 | 0.01 |
| \( C_{\text{av24–48}}/\text{MPC} \) | \( C_{\text{av0–24}}/\text{MPC} \) & 0.02 | 0.01 | 0.03 | 0.01 |
| \( C_{\text{max}}/\text{MPC} \)       | \( C_{\text{max}}/\text{MPC} \) & 0.13 | 0.05 | 0.16 | 0.07 |
| \( T > \text{MPC} \)                | \( h \)       & 0     | 0     | 0     | 0     |

Individual animal pharmacokinetic data for 14 animals divided by mean MPCs for six isolates of each species measured in broth and serum. \( C_{\text{av}} \), average concentration calculated for time periods 0–48, 0–24 and 24–48 h; \( C_{\text{max}} \), maximum plasma concentration (\( \mu g/mL \)); \( T > \text{MPC} \), time for which plasma concentration exceeds MPC (h).
PK/PD modelling

Eight oxytetracycline concentration multiples of MIC, ranging from 0.25 to 8 × MIC, were used in time–kill studies for six isolates each of *A. pleuropneumoniae* and *P. multocida* for 24-h incubation periods. This range established bacteriostatic, bactericidal and 4 log₁₀ reductions in count at 24 h. Breakpoint values of AUC₂₄ h/MIC producing these levels of growth inhibition are presented in Tables 4 (*P. multocida*) and 5 (*A. pleuropneumoniae*).

Dividing the AUC₂₄ h/MIC ratios by 24 yields the concentrations, as MIC multiples, producing bacteriostatic, bactericidal and 4 log₁₀ reductions in count. These are breakpoint KPD values; they are more readily comprehended than the term AUC₂₄ h/MIC. KPDs were 1.10, 2.89 and 4.30, respectively, for *P. multocida* in broth and 1.01, 2.18 and 3.02 for this pathogen in serum. Corresponding values for *A. pleuropneumoniae* were 1.10, 1.64 and 1.89 (broth) and 1.38, 2.31 and 3.32 (serum).

Average dose determination at steady-state

Based on mean CI/F, values for MIC₉₀ for tetracycline (de Jong *et al.*, 2014), the calculated daily doses for bactericidal level of activity were 1748 mg/kg for *P. multocida* and 535 mg/kg for *A. pleuropneumoniae* (Table 5). However, when the worst-case scenario was predicted, using the animal with highest clearance (CI/F = 0.30 L/h/kg) and hence the most rapid clearance, the predicted dose for a bactericidal level of kill was 2381 mg/kg for *P. multocida* and 1269 mg/kg for *A. pleuropneumoniae*.

Dose determination by Monte Carlo simulation

Monte Carlo simulations were conducted using the distribution of CI/F, the distribution of wild-type MICs for tetracycline (de Jong *et al.*, 2014), the tetracycline concentration reducing count to 50% of *P. multocida* and 535 mg/kg for *A. pleuropneumoniae* (Table 6). However, when the worst-case scenario was predicted, using the animal with highest clearance (CI/F = 0.30 L/h/kg) and hence the most rapid clearance, the predicted dose for a bactericidal level of kill was 2381 mg/kg for *P. multocida* and 1269 mg/kg for *A. pleuropneumoniae*.

### Table 4. Pharmacokinetic–pharmacodynamic modelling for *Pasteurella multocida* from time–kill data (mean and standard deviation, n = 6)

| Parameter (units)                  | Broth               | Serum               |
|-----------------------------------|---------------------|---------------------|
|                                  | Mean (CFU/mL)       | Mean (CFU/mL)       |
| Log E₀ (CFU/mL)                   | 2.72 ± 0.30         | 2.28 ± 0.50         |
| Log E_max (CFU/mL)                | −4.52 ± 1.35        | −5.71 ± 1.62        |
| Log E_max – Log E₀ (CFU/mL)       | −7.24 ± 1.05        | −7.98 ± 1.11        |
| Gamma                             | 4.32 ± 0.80         | 2.63 ± 1.40         |
| AUC₂₄ h/MIC for bacteriostatic     | 26.4 ± 7.88         | 24.3 ± 6.66         |
| action (h)                        |                     |                     |
| AUC₂₄ h/MIC₉₀ (h)                 | 33.4 ± 3.84         | 39.9 ± 17.3         |
| AUC₂₄ h/MIC₉₀ for bactericidal     | 69.3 ± 42.8         | 52.3 ± 16.4         |
| action (h)                        |                     |                     |
| AUC₂₄ h/MIC for 4 log₁₀ reduction | 103.3 ± 54.8        | 72.4 ± 26.3         |
| (h)                               |                     |                     |

E₀ = difference in number of organisms (CFU/mL) in control sample in absence of drug between time 0 and 24 h; E_max = difference in number of organisms (CFU/mL) in the presence of oxytetracycline between time 0 and 24 h; AUC₂₄ h/MIC₉₀ = concentration reducing count to 50% of the E_max; Gamma = slope of the curve; detection limit = 33 CFU/mL.

### Table 5. Pharmacokinetic–pharmacodynamic modelling for *Actinobacillus pleuropneumoniae* from time–kill data (mean and standard deviation, n = 6)

| Parameter (units)                  | Broth               | Serum               |
|-----------------------------------|---------------------|---------------------|
|                                  | Mean (CFU/mL)       | Mean (CFU/mL)       |
| Log E₀ (CFU/mL)                   | 2.02 ± 0.37         | 2.46 ± 0.85         |
| Log E_max (CFU/mL)                | −6.48 ± 0.37        | −5.09 ± 2.38        |
| Log E_max − Log E₀ (CFU/mL)       | −8.50 ± 0.00        | −7.45 ± 1.53        |
| Gamma                             | 3.89 ± 0.63         | 3.66 ± 3.02         |
| AUC₂₄ h/MIC for bacteriostatic     | 26.3 ± 2.07         | 33.1 ± 16.7         |
| action (h)                        |                     |                     |
| AUC₂₄ h/MIC₉₀ (h)                 | 35.6 ± 2.74         | 40.1 ± 20.4         |
| AUC₂₄ h/MIC for bactericidal      | 39.5 ± 3.07         | 55.4 ± 19.2         |
| action (h)                        |                     |                     |
| AUC₂₄ h/MIC for 4 log₁₀ reduction | 45.4 ± 4.24         | 79.7 ± 23.6         |

E₀ = difference in number of organisms (CFU/mL) in control sample in absence of drug between time 0 and 24 h; E_max = difference in number of organisms (CFU/mL) in the presence of oxytetracycline between time 0 and 24 h; AUC₂₄ h/MIC₉₀ = concentration reducing count to 50% of the E_max; Gamma = slope of the curve; detection limit = 33 CFU/mL.

### Table 6. Predicted daily doses calculated by deterministic approach

| Daily doses (mg/kg) | *Pasteurella multocida* | *Actinobacillus pleuropneumoniae* |
|---------------------|-------------------------|----------------------------------|
| Bacteriostatic      | 813                     | 268                              |
| Bactericidal        | 1748                    | 535                              |
| 4 log₁₀ count reduction | 2421               | 777                              |

MIC₉₀ for *P. multocida* was 2 µg/ml and 16 µg/ml for *A. pleuropneumoniae* based on tetracycline distribution data (de Jong *et al.*, 2014).
Monte Carlo simulations predicting 50% and 90% target attainment rate dosages at steady-state for three levels of bacterial kill.

Therefore, predicted TAR dosages were even higher for the Japanese MIC oxytetracycline distributions. Thus, for 50% TAR and bacteriostatic doses were 30 mg/kg (broth MIC) and 595 mg/kg (serum MIC), whilst for 90% TAR and a bactericidal level of kill, corresponding doses were 189 and 2237 mg/kg (Table 8).

The single oxytetracycline doses predicted to achieve three levels of kill (0, 3 and 4 log_{10} decreases in count) for three durations of action (24, 48 and 72 h) are presented in Table 9. For a duration of action of 24 h, bacteriostatic levels of kill, the 50% TAR single dosages, using serum/broth pharmacodynamic data for Pasteurella multocida and tetracycline wild-type distribution data (Yoshimura et al., 2001) and using broth and serum MIC distributions and serum/broth MIC data, were 333/17 mg/kg (P. multocida) and 21/25 mg/kg (A. pleuropneumoniae). Bactericidal levels of kill for a duration of action of 48 h at 90% TAR, also using tetracycline MIC distributions and serum/broth MIC data, were 3462/209 mg/kg for P. multocida and 84/76 mg/kg for A. pleuropneumoniae. Doses were higher for P. multocida when predicted using oxytetracycline distribution data (Table 10).

**DISCUSSION**

The objective of this study was to predict doses for oxytetracycline for the pig pneumonia pathogens, P. multocida and A. pleuropneumoniae based on principles of PK/PD integration and modelling and the application of Monte Carlo simulation.

**Pharmacokinetics**

Plasma concentration–time data for oxytetracycline were determined for a small number (14) of healthy pigs of the same age and breed and for two products. In extending the present findings, it will, in future studies, be appropriate to obtain data from greater animal numbers of both sexes and differing ages and breeds and in diseased as well as healthy pigs. Thus, population PK data, derived from field studies, can be used to determine the impact of disease on clearance and bioavailability, CL/F being a major determinant of dosage predictions (Toutain et al., 2017). Nevertheless, the more limited and less variable data used in this study illustrate the principles of integrating PK with PD data to predict TAR dosages.

**Pharmacodynamics**

In this study, a small number of isolates, six for each of two pathogens, were used in PK/PD integration and modelling approaches to dose determination; the question arises whether they are representative of the much larger number of wild-type isolates of these organisms. Clearly, whilst sample size was small, it nevertheless may be noted that: (i) the unavoidable inaccuracy of each individual MIC potency estimate was reduced from potentially approaching 100% (as when single two-dilutions are used using CLSI standards) to not greater than 20% by the use of five overlapping sets of twofold dilutions; (ii) mean broth MICs for oxytetracycline in this study was 2.5 μg/mL (A. pleuropneumoniae), and this may be compared with broth MIC_{50} and MIC_{90} values of 1.0 and 16.0 μg/mL (A. pleuropneumoniae) reported for tetracycline by de Jong et al. (2014). Corresponding values for P. multocida were 0.30 μg/mL (this study) and 0.50 and 2.0 μg/mL for tetracycline (de Jong et al., 2014); and (iii) the present data were supported by a second potency index, namely MPC, and the proportional increase in potency (MPC/MIC ratio) was similar for determinations in both broth and serum for both organisms.

**PK/PD integration**

Pharmacokinetic–pharmacodynamic integration provided an initial and tentative approach for evaluating efficacy of currently recommended oxytetracycline dose schedules for the pathogens, P. multocida and A. pleuropneumoniae. Thus, plasma oxytetracycline \( T > MIC \) values over the period 0–48 h were 11.0 and 15.5 h in broth and serum, respectively, for A. pleuropneumoniae. Corresponding \( T > MICs \) for P. multocida were 52.5 h (broth MIC) and 3.6 h (serum MIC). Therefore, prediction of efficacy from PK/PD integration was broadly similar for potency measured in serum and broth for the former species, but markedly different for the latter pathogen. If it is accepted that potency measured in

| Pathogen | Bacteriostatic | Bactericidal | 4 \( \log_{10} \) reduction |
|----------|----------------|--------------|-----------------|
| Broth    | 30 | 78 | 116 |
| Serum    | 595 | 1453 | 916 |

Monte Carlo simulations predicting 50% and 90% target attainment rate dosages at steady-state for three levels of bacterial kill.

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PK/PD integration and modelling of oxytetracycline

Table 9. Single doses for 24-, 48- and 72-h durations of action based on tetracycline MIC distribution data (de Jong et al., 2014) and using broth and serum pharmacodynamic data

| Dose duration | Level of bacterial kill | Target attainment rate |
|---------------|-------------------------|------------------------|
|               |                         | Broth (50%) | Serum (50%) | Broth (90%) | Serum (90%) |
| **Pasteurella multocida** | Bacteriostatic | 17 | 50 | 333 | 1012 |
| | Bactericidal | 43 | 131 | 716 | 2175 |
| 0–24 h | 4 log₁₀ reduction | 65 | 196 | 992 | 3014 |
| | Bacteriostatic | 25 | 80 | 512 | 1611 |
| | Bactericidal | 67 | 209 | 1102 | 3462 |
| | 4 log₁₀ reduction | 99 | 312 | 1526 | 4796 |
| 0–48 h | Bacteriostatic | 36 | 112 | 719 | 2270 |
| | Bactericidal | 93 | 295 | 1545 | 4879 |
| | 4 log₁₀ reduction | 139 | 439 | 2141 | 6759 |
| 0–72 h | Bacteriostatic | 25 | 93 | 719 | 2270 |
| | Bactericidal | 37 | 121 | 1545 | 4879 |
| | 4 log₁₀ reduction | 67 | 209 | 1102 | 3462 |
| **Actinobacillus pleuropneumoniae** | Bacteriostatic | 25 | 80 | 512 | 1611 |
| 0–24 h | Bactericidal | 37 | 121 | 1545 | 4879 |
| | 4 log₁₀ reduction | 67 | 209 | 1102 | 3462 |
| 0–48 h | Bacteriostatic | 39 | 112 | 719 | 2270 |
| | Bactericidal | 93 | 295 | 1545 | 4879 |
| | 4 log₁₀ reduction | 139 | 439 | 2141 | 6759 |
| 0–72 h | Bacteriostatic | 25 | 93 | 719 | 2270 |
| | Bactericidal | 37 | 121 | 1545 | 4879 |
| | 4 log₁₀ reduction | 67 | 209 | 1102 | 3462 |

Monte Carlo simulations predicting 50% and 90% target attainment rates for three levels of bacterial kill and three action durations.

Table 10. Single doses for 24-, 48- and 72-h durations of action based on oxytetracycline MIC distribution data (Yoshimura et al., 2001) and using broth and serum pharmacodynamic data

| Dose duration | Level of bacterial kill | Target attainment rate |
|---------------|-------------------------|------------------------|
|               |                         | Broth (50%) | Serum (50%) | Broth (90%) | Serum (90%) |
| **Pasteurella multocida** | Bacteriostatic | 42 | 103 | 841 | 2088 |
| | Bactericidal | 109 | 24 | 28 | 1807 | 4487 |
| 0–24 h | 4 log₁₀ reduction | 163 | 404 | 2504 | 6215 |
| | Bacteriostatic | 65 | 159 | 1305 | 3211 |
| | Bactericidal | 169 | 417 | 2804 | 6899 |
| | 4 log₁₀ reduction | 417 | 621 | 3885 | 9557 |
| 0–48 h | Bacteriostatic | 91 | 223 | 1838 | 4503 |
| | Bactericidal | 238 | 584 | 3949 | 9676 |
| | 4 log₁₀ reduction | 355 | 871 | 5471 | 13 403 |
| **Actinobacillus pleuropneumoniae** | Bacteriostatic | 25 | 80 | 512 | 1611 |
| 0–24 h | Bactericidal | 37 | 121 | 1545 | 4879 |
| | 4 log₁₀ reduction | 67 | 209 | 1102 | 3462 |
| 0–48 h | Bacteriostatic | 39 | 112 | 719 | 2270 |
| | Bactericidal | 93 | 295 | 1545 | 4879 |
| | 4 log₁₀ reduction | 139 | 439 | 2141 | 6759 |
| 0–72 h | Bacteriostatic | 25 | 93 | 719 | 2270 |
| | Bactericidal | 37 | 121 | 1545 | 4879 |
| | 4 log₁₀ reduction | 67 | 209 | 1102 | 3462 |

Monte Carlo simulations predicting 50% and 90% target attainment rates for three levels of bacterial kill and three action durations.

As MPCs were 17- to 23-fold greater than corresponding MICs and as MPC:MIC ratios were, moreover, independent of growth matrix, the integration of PK with PD data indicated that plasma concentrations of oxytetracycline could not be achieved, with clinical dosages of oxytetracycline in healthy pigs, which would ensure the eradication of the least sensitive subpopulation in a given colony for both bacterial species.

PK/PD modelling and breakpoint determination

Pharmacokinetic–pharmacodynamic modelling is an advance on PK/PD integration, in that it provides breakpoint values for a given drug against a particular pathogen obtained from a given animal species. In addition, it describes the whole sweep of the concentration–effect relationship, so that any predetermined level of in vitro activity, ranging from bacteriostasis to virtual eradication (indicated by the breakpoint AUC₂₄ h/MIC index) can be determined. In this study, breakpoint values for each level of growth inhibition, 0 log₁₀, 3 log₁₀ and 4 log₁₀ reductions in count, were similar between the two growth matrices; this is not unexpected as, although MICs in broth and serum differed for *P. multocida*, the breakpoint values are based on MIC multiples. However, there were trends for increased interisolate variability with increasing level of kill, especially for *P. multocida* (Tables 4 and 5). This variability might translate to clinical efficacy variability for this pathogen, although the dose determination data imply little or no efficacy from a direct killing action of oxytetracycline (*vide infra*).
Dosage prediction

The deterministic approach to dosage prediction provides a useful estimate of once-daily doses at steady-state, based on $MIC_{90}$ as a single value and the average values for other variables. It does not take into account, however, either of variability or incidence of each input variable, such as clearance or $AUC/MIC$, as dose is calculated using mean values. Nevertheless, it comprises an initial evaluation prior to use of Monte Carlo simulations to estimate population doses for each selected TAR, which is a dose encompassing a given percentile of the target population, for example, 50% or 90% and for three levels of bacterial kill. Moreover, TAR once-daily doses have been determined for maintaining efficacy under steady-state conditions, as well as for TAR single doses over 24, 48 or 72 h time periods, that is either for a single or a loading dose, in the latter case possibly to be repeated after a selected time interval.

The Monte Carlo simulation with PK/PD modelling basis for predicting dosage, for subsequent evaluation in clinical trials, has the advantage of taking into account all important PK and PD variables impinging on bacteriological kill. Furthermore, basing potency estimates on serum as a growth matrix may have greater relevance to in vivo conditions than MIC determined in broths, whilst recognizing that serum, although similar, is not identical in composition to the biophase at infection sites. Moreover, Monte Carlo simulations predict doses, allowing for incidence within MIC distributions and encompassing worst, best and all intermediate case scenarios for distributions of $C/\text{MIC}$ and breakpoint $AUC_{24}$, $\text{MIC}$ ratios.

These clear advantages have led to the widespread application of Monte Carlo simulations to predict dosages (Nielsen et al., 2011; Martínez et al., 2012; Mouton et al., 2012; Brentnall et al., 2013; Nielsen & Friberg, 2013; Papich, 2014; Rey et al., 2014; Sidhu et al., 2014; Lees et al., 2015a; Toutain et al., 2017). Nevertheless, there are inevitable limitations to study methodology. For example, for this study MIC distribution data were extracted from the literature, and the number of isolates was limited (230 for $P.\text{multocida}$ and 220 for $A.\text{pleuropneumoniae}$). Moreover, they were obtained from one geographical location (Europe) for tetracycline, a drug with likely similar but not necessarily identical antimicrobial profile to oxytetracycline. For CLSI (2013), tetracycline is the class representative but it is proposed that MIC tetracycline distribution also applies to oxytetracycline. Comparing tetracycline MIC distribution for European isolates against Japanese oxytetracycline MIC distribution for $P.\text{multocida}$ indicated MIC ranges of 0.2–12.5 $\mu\text{g/mL}$ for oxytetracycline (including likely resistant isolates) and 0.25–32 $\mu\text{g/mL}$ for tetracycline (including likely resistant isolates). $MIC_{50}$ and $MIC_{90}$ differed, 1.56 and 12.5 $\mu\text{g/mL}$ respectively, for oxytetracycline, and corresponding values for tetracycline were 0.5 and 2.0 $\mu\text{g/mL}$.

Whilst the interisolate variability in PK/PD breakpoint values was small to moderate in the present study, estimates were based on only six isolates for each species. The time–kill studies used fixed drug concentrations (eight multiples of MIC) for a fixed time period. In clinical use, on the other hand, plasma drug concentrations would first increase and then decrease after intramuscular dosing, exposing organisms to a continuously variable concentration. In future studies, these concerns could be addressed by increasing numbers of isolates in field distribution studies and in PK/PD breakpoint estimation studies. Moreover, exposure of organisms to varying drug concentrations could be addressed by use of in vivo pump (e.g. hollow fibre) methods to simulate in vitro patterns of change in concentration with time (Cadwell, 2012).

Finally, the methodology in this study also does not incorporate the contribution to pathogen elimination by the body’s natural defence mechanisms in immune-competent clinical subjects, nor does it allow for additional potentially beneficial properties of antimicrobial drugs, such as immunomodulatory and anti-inflammatory actions (vide infra).

The depot formulations of oxytetracycline licensed to treat porcine respiratory diseases recommend a single dose of 20 mg/kg to achieve up to four days action duration. Based on serum MICs, the predicted 90% TAR dosage against $P.\text{multocida}$ was 1123 mg/kg daily (bactericidal action at steady-state) and 3462 mg/kg (bactericidal action as single dose and 48-h duration). In similar studies for $P.\text{multocida}$ calf origin, corresponding doses were 921 and 1523 mg/kg (Lees et al., 2016). These differences for the two animal species and the same bacterial species are explained by: similar values for the $AUC_{24}$, $\text{MIC}$ bactericidal breakpoints: approximately twofold higher values of $C/\text{MIC}$ in the pig; differing free drug fractions in serum (29% in the pig, 48% in the calf); and differing MIC distribution patterns. For both animal species, however, it is clear that practicable doses to achieve a direct killing action of oxytetracycline are likely not to be attainable.

For $A.\text{pleuropneumoniae}$, predicted dosages for 90% TAR and bactericidal level of kill were 42.5 mg/kg (daily dose at steady-state) and 84.3 mg/kg (single dose with 48-h duration). Whilst still exceeding the recommended dose regimen, these lower doses for this pathogen compared to $P.\text{multocida}$ reflect the broth:serotiny differences for the two bacterial species. Serum:broth MIC ratios, after correcting for protein binding in serum, for six isolates of each species were 6.30:1 ($P.\text{multocida}$) and 0.24:1 ($A.\text{pleuropneumoniae}$) (Dorey et al., 2016).

In conclusion, it might be noted that tetracyclines have activities other than direct killing actions, and clinically, these might contribute to or even account for efficacy.

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