Standard PK/PD concepts can be applied to determine a dosage regimen for a macrolide: the case of tulathromycin in the calf

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Standard PK/PD concepts can be applied to determine a dosage regimen for a macrolide: the case of tulathromycin in the calf

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Short running title: PK/PD for dosage regimen for tulathromycin in calf

Key words: Tulathromycin; PK/PD; dosage regimen; Monte Carlo simulation

Abstract

The pharmacokinetic (PK) profile of tulathromycin, administered to calves subcutaneously at the dose rate of 2.5 mg/kg, was established in serum, inflamed (exudate) and non-inflamed (transudate) fluids in a tissue cage model. The PK profile of tulathromycin was also established in pneumonic calves. For *Mannheimia haemolytica* and *Pasteurella multocida*, tulathromycin Minimum Inhibitory Concentrations (MIC) were approximately 50 times lower in calf serum than in Mueller Hinton Broth. The breakpoint value of the PK/pharmacodynamic (PD) index ($AUC_{(0\text{-}24h)}$/MIC) to achieve a bactericidal effect was estimated from *in vitro* time-kill studies to be approximately 24 h for *M. haemolytica* and *P. multocida*. A population model was developed from healthy and pneumonic calves and, using Monte Carlo simulations, PK/PD cut-offs required for the development of Antimicrobial Susceptibility Testing (AST) were determined. The population distributions of tulathromycin doses were established by Monte Carlo Computation (MCC). The computation predicted a Target Attainment Rate (TAR) for a tulathromycin dose rate of 2.5 mg/kg of 66% for *M. haemolytica* and 87% for *P. multocida*. The findings indicate that free tulathromycin concentrations in serum suffice to explain the efficacy of single dose tulathromycin in clinical use, and that a dosage regimen can be computed for tulathromycin using classical PK/PD concepts.

Introduction

Good clinical efficacy and bacteriological cure with macrolides in human and animal medicine are commonly achievable with plasma/serum concentrations that are lower, even much lower, than the *in vitro* MICs for major lung pathogens. This is the case

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for tulathromycin (Nowakowski, Inskeep et al., 2004), gamithromycin (Huang, Letendre et al., 2010) and tildipirosin (Menge, Rose et al., 2012), three agents of the triamilide sub-class, licensed for farm animal use. Because of the disparity between *in vivo* plasma concentration and *in vitro* MICs, the application of classical PK/PD concepts to macrolides has frequently been challenged. This has led some authors and regulatory authorities to claim that there is no plasma concentration-effect relationship for macrolides. A corollary has been to propose that dosages for clinical use can only be established in a clinical setting using a dose-titration approach. However, an alternative to this view is to question the value and applicability of the *in vitro* MIC data obtained in matrices optimized for bacterial growth, as for example in Mueller Hinton Broth (MHB). This issue is addressed in this article.

To explain the clinical efficacy of macrolides at recommended dose rates, alternatives to the PK/PD paradigm have been proposed. For example, many authors have proposed that lung tissue concentration is more relevant than that in plasma/serum, in determining outcome of treatment with macrolides. For tulathromycin, the drug investigated in the present paper, lung homogenate concentrations in pigs and calves were more than 50-fold higher than corresponding plasma concentrations (Nowakowski, Inskeep et al., 2004; Villarino, Lesman et al., 2013). However, it is now widely accepted that lung homogenate is not the biophase for lung infections (Mouton, Theuretzbacher et al., 2008; Villarino, Brown et al., 2013; Villarino, Lesman et al., 2013; Villarino, Lesman et al., 2013; Villarino, Brown et al., 2014). The two pathogens considered in this article, *Pasteurella multocida* and *Mannheimia haemolytica*, are strictly extracellular pathogens and pulmonary epithelial lining fluid (PELF) is the main location for such extracellular organisms. In some studies, PELF concentrations have exceeded the plasma non-protein bound

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concentrations, leading to a view of the lung as a local drug "reservoir", able to control the local extracellular concentration. However, *in vivo* conditions are dynamic and any (very slow) release of drug from the lung (very long half-life) would be unable to maintain an effective local extracellular concentration. Consequently, the lungs should, rather, be viewed not as a "reservoir" but as a "sump". According to Kiem and Schentag (Kiem & Schentag, 2008), it is the lysis of cells (containing high drug concentrations) during the bronchoalveolar lavage procedure required to collect PELF that explains high PELF drug concentrations. Therefore, these authors, consider the high drug concentrations in PELF to be artefactual.

Many reports have shown that macrolides may accumulate in high concentrations in neutrophils and macrophages (Scorneaux & Shryock, 1998; Scorneaux & Shryock, 1999; Villarino, Brown et al., 2013). Uptake by and subsequent off-loading of drug, for example azithromycin, from these cells *in vivo* has been proposed as a mechanism of drug delivery to the biophase (Gladue, Bright et al., 1989; Mandell & Coleman, 2001; Bosnar, Kelneric et al., 2005). However, *in vivo* evidence to support this hypothesis is entirely lacking and it may also be questioned on theoretical grounds using mass balance considerations. Even assuming that the entire circulating neutrophil pool is in the lung extracellular water (about $2x10^{11}$ cells corresponding to a volume of 6mL for a 50kg calf) and also that the total macrolide content is immediately released, the neutrophil pool would be unable to dynamically control the local biophase concentration over the several days of the claimed duration of drug efficacy. We conclude that it is most improbable that these cells can be accorded the role of "truck-containing bullets off-loading their drug content" and targeting the biophase, thereby achieving *in vivo* high local and sustained drug concentrations (Toutain, 2009). This opinion is consistent with the fact that, using

microdialysis techniques, it has been shown that inflammation generally does not increase local antimicrobial drug (AMD) concentrations (Muller, dela Pena et al., 2004).

In light of the challenges to the applicability of the PK/PD paradigm to macrolides, the aim of this study was to generate PK and PD data for tulathromycin for *M. haemolytica* and *P. multocida* in calves to show that it *is* possible to establish therapeutically relevant *in vivo* PK/PD relationships for a macrolide, as for any AMD (Toutain, del Castillo et al., 2002; Toutain & Lees, 2004). More specifically, using a population PK model of tulathromycin disposition, we have estimated PK/PD cut-offs of tulathromycin to compare their Target Attainment Rate (TAR) with the current clinical breakpoints (BP) used for Antimicrobial Susceptibility Testing (AST) in cattle. Finally, using MIC distributions of *M. haemolytica* and *P. multocida*, we have generated by Monte Carlo computation (MCC) the population distribution of the tulathromycin doses to determine the corresponding TAR for the currently marketed dose of 2.5 mg/kg. This has provided a comparison of results obtained using a PK/PD approach with the dose derived from the dose titration approach.

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Materials and Methods

Animals and procedures

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Ethics and Welfare Committee of the Royal Veterinary College and the UK Home Office (Project License 70/6986) and all efforts were made to minimize suffering.

A PK study was conducted in 10 healthy female Aberdeen Angus calves and 16 calves that were subjected to an experimental pulmonary infection. For healthy calves, body weights were in the range 145-204 kg and ages ranged from 79-131 days. Tissue cages were implanted subcutaneously in the paralumbar fossa, under general anesthesia, as described by Sidhu et al. (Sidhu, Shojaee Aliabadi et al., 2003). Tulathromycin (Draxxin®, Pfizer Animal Health, Sandwich, Kent, UK) was injected subcutaneously into the flank at a dose rate of 2.5 mg/kg at zero time. Also at zero time, 0.5 mL of 1% w/v sterile lambda carrageenan solution in saline (Viscarin, Marine Colloids, Springfield, Ill, U.S.A.) was injected into a single tissue cage. This cage was used to harvest exudate. A second, unstimulated cage was used to collect non-inflammatory fluid (transudate).

A pneumonia model using *M.haemolytica* type A1; ref M 7/2 was used in 16 two-week old Holstein Friesian bull calves. Upon arrival, animals were treated with a single subcutaneous dose of florfenicol 40 mg/kg bodyweight (Nuflor, Schering-Plough Animal Health, Middlesex, UK), to ensure freedom from sub-clinical infections. Animals were weaned at 5-6 weeks of age, after which they were group housed until the start of the study. The calves were 12-13 weeks of age at the commencement of the study. A *M. haemolytica* inoculum containing 1.27-9.60 10^9 cfu/mL was used to induce pneumonia as previously described (Sarasola, Jernigan et al., 2002).

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121 **Sampling procedures**

122 For healthy calves, blood samples (10 mL volume) were collected, protected from
123 light, from a jugular vein, into vacutainers (Becton, Dickinson and Company, Oxford,
124 Oxon, U.K.) without anticoagulant, before and regularly up to 336 h after injection of
125 tulathromycin. Exudate and transudate samples (1.5 mL volume) were collected,
126 protected from light, before and regularly up to 336 h after dosing. All samples were
127 centrifuged (2000 g for 10 min at 4°C) and supernatants stored at -70°C prior to
128 assay for tulathromycin concentration and for measurement of *ex vivo* antibacterial
129 activity. For experimentally infected calves, blood samples were collected regularly
130 from time 0 to 48 h after injection of tulathromycin and samples were processed as
131 described for the healthy calves.

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133 **Analysis of tulathromycin in serum, exudate and transudate**

134 Serum, exudate and transudate samples were assayed for tulathromycin, using a
135 tandem mass spectrometry method, adapted from Scheuch *et al*. (Scheuch, Spieker
136 et al., 2007).

137 The methods were validated according to the published guideline (Committee for
138 medicinal products for human use (CHMP), 2011). Selectivity was checked for each
139 matrix. A quadratic model weighted by $1/X^2$ was selected over the calibration range
140 5-1000 ng/mL. Intra-day and inter-day precisions were less than 15% and the
141 accuracy ranged from 102 to 106%. The lower limit of quantification (LLOQ) was 5
142 ng/mL with a precision of 8% and an accuracy of 105%. The processed extracts of
143 tulathromycin were stable in the autosampler at room temperature for at least 24 h.

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3 145 **Pharmacokinetic-pharmacodynamic modelling of *in vitro* time-kill data**
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5 146 PK/PD modelling of *in vitro* time-kill data was undertaken to compute the BP value of
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7 147 the PK/PD index ($AUC_{(0-24h)}$/MIC) selected to express tulathromycin *in vivo* activity
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9 148 i.e. its bacteriostatic, bactericidal or 4 log_{10} decrease in count potency. Data from
10
11 149 previously reported time-kill studies, using both MHB and calf serum as matrices,
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13 150 were used for PK/PD modelling (see for details (Lees, Illambas et al., 2016)). Briefly,
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15 151 six isolates each of the species *M. haemolytica* and *P. multocida* were investigated,
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17 152 using tulathromycin concentrations corresponding to 0.25, 0.5, 1, 2 and 4 x multiples
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19 153 of MIC. The bacterial growth, after 24 h incubation in the absence or presence of a
20
21 154 given tulathromycin concentration (expressed as log_{10} cfu/mL subtracted from the
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23 155 initial inoculum count log_{10} cfu/mL), was measured as the response to the
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25 156 tulathromycin *in vitro* activity (dependent variable); the ratios of $AUC_{(0-24h)}$/MIC were
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27 157 calculated for each isolate at each of the five tulathromycin concentrations tested and
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29 158 these comprised the independent variable for fitting data to a sigmoidal E_{max} model to
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31 159 estimate parameters of tulathromycin efficacy and potency (Equation 1).
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$$E = E_0 + \frac{E_{max} \times C^N}{EC_{50}^N + C^N} \quad (1)$$
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42 161 where E is the bacterial growth for a given concentration of tulathromycin; E_0 is the
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44 162 corresponding bacterial growth in the absence of drug (control samples); E_{max} (the
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46 163 efficacy parameter) is the maximum antimicrobial growth inhibition determined as the
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48 164 change in log_{10} cfu/mL over 24 h incubation; EC_{50}^N (the potency parameter) is the
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50 165 $AUC_{(0-24h)}$/MIC value providing 50% of the maximum antibacterial effect; *C* (the
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52 166 independent variable) is the concentration term (expressed as $AUC_{(0-24h)}/MIC$); and *N*
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54 167 is the Hill coefficient. The $AUC_{(0-24h)}$/MIC corresponding to a bacteriostatic effect
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168 (*E=0*, no change form initial inoculum count), a bactericidal effect (*E=-3*, a $3\log_{10}$
169 reduction from initial inoculum count) or (*E=-4* a $4\log_{10}$ reduction from initial inoculum
170 count) were computed by solving equation 1 for each isolate of each organism in
171 MHB and serum (Aliabadi & Lees, 2001; Lees, Shojaee Aliabadi et al., 2004).

172

173 **Pharmacokinetic analyses**

174 Tulathromycin concentration-time data in serum, exudate and transudate in individual
175 calves were analyzed using the WIN-NONLIN regression program (Pharsight
176 Corporation, Mountain View, CA, USA) submitted to non-compartmental analysis
177 using the statistical moment approach.

178 A more advanced population analysis was carried out with Phoenix (Phoenix
179 WinNonlin 6.3 and NLME1.2; Certara, L.P., St. Louis, MO, USA) to analyze
180 simultaneously the serum concentrations obtained from the 10 control calves and the
181 16 calves subjected to an experimental infection. The more specific goal of the
182 population analysis was to develop a basic model of tulathromycin disposition in
183 calves, in order to compute the PK/PD cut-off values that could be used
184 subsequently to select a clinical BP for an AST that will be adapted to the calf. As the
185 BP for any AST is always a single value (for a given species and a given pathogen) a
186 PK model including different co-variates was not required for this objective.
187 Nevertheless, we explored the health status (pneumonic vs. control calves) as a
188 relevant co-variate to assess the influence, if any, of an experimentally induced
189 pneumonia on tulathromycin disposition in the calf.

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190 The selected structural model was a bi-exponential model for an extravascular route
191 of administration. The parameterisation was carried out in terms of macroconstants
192 corresponding to equation 2:

193 $$Y(t) = A \times EXP(-\alpha \times t) + B \times EXP(-\beta \times t) - (A+B) \times EXP(-KA \times t) \quad (2)$$

194 Where *A* and *B* (ng/mL) are intercepts and *Ka, Alpha* and *Beta* are slopes (1/h) with
195 *Ka>alpha>beta*. The estimated fixed parameters (the thetas vector) are reported as
196 their typical values (*tv*). The second component of a mixed effect model is the
197 random component (random effect). The Between Subject Variability (BSV) was
198 modeled using an exponential model of the form:

199 $$\theta_i = \theta_1 \times EXP(\eta_i) \quad (3)$$

200 where θ_i is the value of theta in the ith animal, θ_1 is the typical population value of
201 this theta and η_i, the deviation (noted eta) associated to the ith animal from the
202 corresponding theta population value. The distribution of the etas was assumed to
203 be normal with a mean of 0 and a variance ω^2.

204

205 A full covariance matrix of the *etas* was estimated. From the diagonal of this matrix,
206 formed by the variance terms in the log-domain, we estimated the BSV using
207 equation 4 which converts the variance to a coefficient of variation (CV%) in the
208 original scale.

209 $$CV(\%) = 100 \times \sqrt{EXP(\omega^2) - 1} \quad (4)$$

210 The full matrix of the etas was used for the subsequent Monte Carlo simulations.

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3 211 The residual model which reflects unexplained variability after controlling for other
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5 212 sources of variability (analytical imprecision, departure from the model) was a
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7 213 multiplicative (proportional) model with ε the error term having a mean of 0 and a
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9 214 variance $\sigma 2$.
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12 215 Parameter estimation with associated standard errors (SE), as a measure of the
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14 216 precision of the estimation, was based on minimizing an objective function value
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16 217 using maximum likelihood estimation. A Quasi-Random Parametric Expectation
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18 218 Maximization (QRPEM) was selected for this estimation. Shrinkage of random effects
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20 219 towards the means was calculated for the etas and epsilon, allowing use of Empirical
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22 220 Bayes Estimates -based diagnostic (Karlsson & Savic, 2007).
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24
25 221 Monte Carlo simulations with the basic model were performed to generate 1,040 data
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27 222 sets from 0 to 336 h with a step of 6 h. These simulated serum tulathromycin
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29 223 concentration profiles were used to determine the PK/PD cut-off values and the
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31 224 distribution of the apparent clearance, which was used for the population dose
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33 225 computation.
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39 227 **Population Dosage prediction**
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41 228 The adequacy of the current dosage of tulathromycin (2.5 mg/kg) was explored by
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43 229 computing population doses covering different TAR i.e. different percentages of the
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45 230 population to assess the ability of a PK/PD model to validate or not the current dose
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47 231 for tulathromycin. For AMDs for which the PK/PD index that best predicts efficacy is
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49 232 $AUC_{(0\text{-}24h)}/MIC$, the following equation can be used (Equation 5).
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$$Dose_{(\text{for 10 days of activity})} = \frac{Cl_{\text{for 10 days}} \times SF \times MIC_{distribution}}{fu \times F} \quad (5)$$

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234 Where $Dose$ is the amount of AMD to be administered to guarantee an activity of
235 tulathromycin over 10 days for a given TAR (see Discussion for the selection of this
236 10 days duration); $Cl_{\text{for 10 days}}$ is the population distribution of the apparent plasma
237 clearance (Cl/F) as obtained in our population PK analysis; SF is a scaling factor
238 without units, obtained by dividing the BP value of the PK/PD index i.e. $AUC_{(0-24h)}/MIC$, by 24 h, permitting computation of a SF of 1 for the present analysis; this
240 indicates that, to achieve a bactericidal action, the average serum concentration of
241 tulathromycin should be equal to the MIC (see (Toutain, Bousquet-Melou et al., 2007)
242 for further explanation on derivation of the SF from $AUC_{(0-24h)}/MIC$); fu is the free
243 drug fraction (Anonymous, 2005) (the binding to serum protein is low, with the bound
244 fraction ranging from 0.32 to 0.39 in cattle) and we incorporated in equation 5 fu as
245 a uniform distribution between 0.61 and 0.68. For the MIC distributions, the results of
246 a survey kindly provided by J. Watts (Zoetis) giving the MIC distribution of *P.
247 multocida* and *M. haemolytica* from the years 2004 to 2010, obtained in artificial
248 broth, were used. We first selected all the MICs equal to or less than the BP
249 proposed by the CLSI i.e. 16 µg/mL; this value is, according to CLSI, the susceptible
250 pathogen population that clinically can be successfully treated with tulathromycin and
251 that should therefore be considered to compute a dose. The percentage of
252 susceptible pathogens was 89.82% and 91.11% for *M.haemolytica* (n=2233 strains)
253 and *P. multocida* (n=2483 strains), respectively. The MHB MICs were then
254 transformed into vectors of equivalent MICs in serum by dividing all reported MICs
255 by a factor of 50 i.e. by the SF previously determined when comparing MICs in the
256 two matrices (Lees, Illambas et al., 2016).
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258 Dosage distribution was computed using MCC (n=5000) in Oracle Crystal Ball
259 (Oracle Corporation, Redwood Shores, CA, USA) and the range of TAR from 0 to
260 90% of cases for which a range of dose levels are able to achieve an average serum
261 concentration equal to the MIC over the 10 days. The dose level corresponding to a
262 TAR of 90% was considered as the population dose of tulathromycin, given the
263 actual susceptible MIC distribution and assuming activity duration of 10 days. Finally,
264 a sensitivity analysis was carried out to apportion the overall variability of the dose
265 distributions either to the PK variables (clearance, fu) or to the PD variability (MIC).
266 Figure 1 gives a flowchart of the different steps for data collection and analysis.
267 **Fig.1**
268

269 Statistical analyses

270 PK variables are presented as geometric, harmonic or arithmetic means, as
271 appropriate, and SEM. Mean differences in $AUC_{(0\text{-}24h)}$/MIC ratios determined in MHB
272 compared with those determined in serum for bacteriostatic, bactericidal and $4\log_{10}$
273 reductions in count were compared by ANOVA.
274

275 Results

276 Calf *in vivo* study

277 *Tulathromycin concentrations in serum, exudate and transudate (NCA*
278 *analysis)*

279 The mean (+SEM) concentrations of tulathromycin are presented in Fig. 2 (serum, 0-
280 15 h) and Fig. 3 (serum, exudate and transudate, 0-336 h). Maximum concentration
281 (C_{max}) of tulathromycin (0.69 µg/mL) was often observed at the first sampling time (10
282 min). Concentrations exceeded 0.40 µg/mL up to 12 h and were still quantifiable

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(mean = 0.025 μg/mL) at 336 h. Serum terminal half-life was 84.0 h and mean residence time (MRT) was 91.4 h (Table 1).

Fig. 2

Fig. 3

Penetration of tulathromycin into and clearance from carrageenan-induced exudate and transudate were dissimilar. Concentrations (C_{max}) and areas under curves ($AUC_{(0\text{-last})}$) were higher in exudate than in transudate; 0.24 and 0.12 μg/mL for C_{max} ($P<0.05$) and 18.4 and 8.58 μg.h/mL for $AUC_{(0\text{-last})}$ ($P<0.05$). More importantly , for both non-vascular fluids concentrations and areas were lower than in serum at all sampling times, $P<0.01$ (for serum C_{max} = 0.75 μg/mL and $AUC_{(0\text{-last})}$ = 45.1 μg.h/mL) indicating a lack of tulathromycin accumulation in an inflammatory fluid compared to serum (see discussion). However, mean values of MRT were similar for all three fluids, indicating that a state of equilibrium was achieved between the tissue cages and serum.

Pharmacokinetic-pharmacodynamic surrogates

PK/PD integration established the surrogates, C_{max}/MIC, T>MIC, $AUC_{(0\text{-}24h)}$/MIC and $AUC_{(0\text{-}\infty)}$/MIC for tulathromycin, derived from *in vivo* concentrations in the PK study (n=10) and *in vitro* MICs (n=6 for each species) measured in serum. Based on the geometric mean serum MICs of 0.04 μg/mL for both *P. multocida* and *M. haemolytica*, C_{max}/MIC (18.75±0.11), $AUC_{(0\text{-}24h)}$/MIC (238±5.8h), $AUC_{(0\text{-}\infty)}$/MIC ratios (1198±6.3h) and T>MIC (281±17.5h) indicated that serum concentrations of

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306 tulathromycin would be predicted to have a high level of activity against the six
307 strains each of *P. multocida* and *M. haemolytica* investigated.

308 **In vitro PK/PD modelling to establish the breakpoint value of $AUC_{(0-24h)}$/MIC**

309 The BP value of the PK/PD index was required to compute a dose (SF of equation
310 5); for both *M. haemolytica* and *P. multocida* geometric mean MIC values were 2.07
311 µg/mL (n = 6 per species) in MHB and 0.04 µg/mL (n = 6) in calf serum (Lees,
312 Illambas et al., 2016). For both matrices and both organisms, the *in vitro* time-kill data
313 provided typical BP estimates of $AUC_{(0-24h)}$/MIC required to produce, after 24 h
314 exposure, three levels of growth inhibition (Table 2). A strong bactericidal effect was
315 observed with a steep concentration-effect relationship. For both *M.haemolytica* and
316 *P multocida* $AUC_{(0-24h)}$/MIC values providing a $3log_{10}$ reduction in count were
317 approximately 24 h in both serum and MHB. The same result expressed in term of
318 multiples of MIC was approximately 1 and we selected for the MCS an overall
319 average BP of 24 h for the PK/PD index (*i.e.* a SF equal to 1 in equation 5).

320 The reported AUC/MIC values (in h) are proportionality factors between the
321 experimental MIC (MICexp) and the average MHB or the average serum
322 tulathromycin concentration to achieve a bacteriostatic or a bactericidal effect e.g.
323 the $AUC_{(0-24h)}$/MIC of 17.15 h providing a bacteriostatic effect in MHB indicates that
324 the corresponding average concentration over 24 h in MHB was
325 17.15h/24h=0.71times the MIC and that the bactericidal effect in serum was obtained
326 with a concentration of 38.44h/24h or 1.6 times the MIC.

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3 328 **Population analysis**
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6 329 Figure 4 shows the Visual Predictive Check (VPC) *i.e.* the observed serum
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8 330 concentration (ng/ml) vs. time (h) and observed and predicted quantiles; this is the
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11 331 most useful diagnostic plot for the assessment of model adequacy. Typical values of
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13 332 the primary structural parameters of the model (thetas), the secondary parameters
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15 333 (AUC and half-life), their associated Standard Error (SE) and the SD of the residual
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17 334 for the basic model are given in Table 3; for all calves, the *tv* of the terminal half-life
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19 335 was 84.8 h (CV 4.8%) and the *tv* of the population AUC was 46,291 ng*h/mL (CV
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21
22 336 11.5%) *i.e.* very similar values to those computed by the NCA with the 10 control
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24 337 calves. The estimates of shrinkage were relatively small or moderate and provided
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26 338 support for the reliability of the estimates of the random effects. Coefficient of
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28 339 variation (CV%) of the variance of the etas expressing the BSV are given in Table 4.
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31 340 They were relatively large for A, alpha and Ka and the inclusion in the model of the
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33 341 covariate health status largely reduced these CV% (Table 4).
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36 342 A and B (ng/mL) are intercepts and Ka, Alpha and Beta are slopes (1/h) with
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38 343 Ka>alpha>beta. CV% was the standard error percentage calculated as 100* SE /
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41 344 Parameter Value indicating the reliability of the model. Proportional residual error
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43 345 was 24.5% and the epsilon shrinkage was 0.05375.
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45 346 **Fig.4**
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49 348 Using the basic structural model, the serum disposition curves (Individual Predicted
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52 349 values based on individual ETAs) of 1,040 calves were simulated (serum
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54 350 concentrations predicted from time 0 to 336 h with a step of 6 h). These 1,040
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56 351 simulations were subjected to a NCA, and partial areas (from 0 to 24h, 0 to 48h…up
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58 352 to 0 to 240h) were computed; then the corresponding mean serum concentrations
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353 over these time intervals were calculated. Next, the corresponding quantiles (5 to
354 95%) were established (Table 5). These serum concentrations can be viewed as the
355 PK/PD cut-offs for these time intervals and for a selected TAR. For example, if the
356 claimed duration of tulathromycin activity is 240 h and if a TAR of 90% is expected,
357 the PK/PD cut-off is 83 ng/mL, indicating that for 90% of the calves it can be
358 guaranteed that the average serum concentration of tulathromycin will be at least 83
359 ng/mL over the first 10 days following tulathromycin administration at the nominal
360 dosage of 2.5mg/kg. As the ratio of MIC obtained in MHB:serum is approximately
361 50:1, the PK/PD cut-off for a TAR of 90% for an expected duration of activity of 240 h
362 is 4.15 µg/mL for MHB, a value lower than the current clinical breakpoint of CLSI (16
363 µg/mL) (Lees, Illambas et al., 2016) but consistent with EMEA opinion (Committee for
364 medicinal products for veterinary use (CVMP), 2003) (see Discussion).
365

366 **Dosage computation**

367 From the MCS, using parameters of the basic model, the corresponding 1,040
368 apparent serum clearance values were computed (Dose/$AUC_{(0\text{-inf})}$) with Dose = the
369 standard tulathromycin dose of 2.5mg/kg. The distribution of these 1,040 serum
370 clearances (L/kg/day) was right-skewed and this distribution was normalized by a log
371 transformation (log of the mean clearance of 0.287 and SD of 0.53252). The mean
372 clearance for the 10 days selected for solving equation 5 was 2.589 in the LN
373 domain.The distribution of MICs, after transformation by a scaling factor of 50 to take
374 into account the serum effect, is presented in Figure 5.
375 Figures 6 and 7 illustrate the non-cumulative and cumulative distributions of doses
376 for *M. haemolytica* and *P. multocida,* respectively. For a TAR of 90%, the computed
377 dose for *M. haemolytica* was 5.3mg/kg, indicating that, with this dose, the average

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378 serum concentration of tulathromycin over the first 240 h following administration of
379 this dose will be equal to the MIC of *M. haemolytica* in 90% of calves. For the
380 nominal dose of 2.5mg/kg, the corresponding TAR *for M. haemolytica was* 65.9%.
381 For a TAR of 90%, the computed dose for *P. multocida* was 2.52 mg/kg and for the
382 nominal dose of 2.50 mg/kg, the TAR was 87.2%.

383 **Fig. 5**

384 **Fig 6**

385 **Fig. 7**

386 To identify the main sources of variability amongst the factors controlling the dose
387 (plasma clearance, MICs or fu), a sensitivity analysis was performed to apportion
388 variability to these three factors. The main source of variability for *P. multocida* was
389 MIC (76.7%); the BSV of serum clearance was lower (23.0%) and the variability of *fu*
390 was negligible. For *M. haemolytica*, results were very similar with 70.3% of the
391 variability due to the MIC distribution and 29.2% to the BSV of clearance, indicating
392 that the data should be relevant for different cattle populations.

393

394 Discussion

395 The principal findings of the present study are that: (i) contrary to previous claims, the
396 dosage of tulathromycin can be documented using standard PK/PD concepts; (ii) the
397 recommended dose of tulathromycin (2.5mg/kg) is consistent with our calculated
398 population doses, with computed TARs of 66% and 87% for a 2.50 mg/kg dose for *M.*
399 *haemolytica* and *P. multocida,* respectively; (iii) tulathromycin did not accumulate in
400 inflammatory exudate in comparison with serum concentrations; (iv) the BP value of

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3 401 the PK/PD index *i.e.* the $AUC_{(0-24h)}$/MIC was approximately 24 h, indicating that
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5 402 tulathromycin has a potent bactericidal effect for a concentration approximately equal
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7 403 to the MIC; (v) PK/PD cut-offs are consistent with the current BPs of AST issued by
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9 404 the CLSI and EMA; and (vi) the main source of variability to take into account to
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11 405 determine a dose for a given animal is of PD origin (MIC) and not the actual
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13 406 tulathromycin exposure.
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18 408 The aim of this study was to determine the adequacy (or not) of the currently
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20 409 recommended dosage of tulathromycin (2.5mg/kg) for calves, by computing a
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22 410 population dose covering a range of TARs *i.e.* the aim was to assess the ability of a
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24 411 standard PK/PD model to determine a dose for tulathromycin for different
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26 412 percentages (up to 95%) of the population. The present PK/PD approach clearly
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28 413 indicated the therapeutic relevance of serum concentration; and the data further
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30 414 suggest that other explanations for the efficacy of tulathromycin, including high local
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32 415 intracellular pulmonary concentration or a specific transportation of the drug by
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34 416 macrophages or neutrophils to the extracellular biophase in the lung (as discussed in
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36 417 the Introduction) do not need to be invoked. The dose of tulathromycin was first (prior
37
38 418 to licensing) determined in field studies and was based primarily on clinical efficacy,
39
40 419 because it was considered that the clinical efficacy for this drug could not be derived
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42 420 from a PK/PD relationship. The present data provide clear evidence that standard
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44 421 PK/PD concepts would, in fact, have fully sufficed to derive an appropriate dosage for
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46 422 tulathromycin. The crucial proviso, however, is that, for the PK/PD integration and
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48 423 modelling approaches to be valid, MICs have to be determined in serum and not, as
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50 424 is routine and almost universal, in an artificial medium such as MHB. We therefore
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52 425 conclude that what should be questioned for macrolides is the utilization of *in vitro*
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MICs in a non-biological fluid such as MHB, when establishing PK/PD relationships as a basis for dosage determination.

The main limitation to our computations is the selected value of the SF used to transform MICs derived from the epidemiological survey and obtained in broth, into equivalent MICs in serum. A factor of 50 was used, based on broth:serum MIC ratios for tulathromycin reported in our previous paper (Lees, Illambas et al., 2016). This revealed a very large serum effect on the potency of tulathromycin, as had been previously reported by others (Godinho, Keane et al., 2005). For example, Godhino et al (Godinho, 2008) obtained a lower broth:serum MIC ratio (up to 16) but in the presence of only 40% bovine serum (rather than the 100% serum used in the present study).

As the ratio MHB:serum MIC is approximately 50, the PK/PD cut-off (in MHB) for a TAR of 90% for an expected duration of activity of 240 h is 4.15 μg/mL, a value almost four times lower than the current clinical BP of the CLSI (16 μg/mL) but more consistent with the EMA/CVMP BP of ≤ 8 μg/mL (Committee for medicinal products for veterinary use (CVMP), 2003) supporting the validity of our PK/PD approach.

The second possible limitation of our computations is the limited number of animals that were considered in the population analysis (n=26) with the possibility of having excluded some relevant variability factors (for example breed of animal). Nevertheless, we are confident in the overall conclusions, because the sensitivity analysis showed clearly that the impact of the PK variability is minimal in comparison with that of PD origin (the epidemiological MICs). Moreover, our population model included both healthy and diseased animals and it was previously reported that there were no statistically significant differences in tulathromycin PK between pre-ruminant calves and adult cattle (Committee for medicinal products for veterinary use (CVMP),

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451 2003). It is concluded that our PK population model was sufficiently robust for the
452 purpose of deriving PK/PD cut-offs and population dose distributions.

453 For single dose administration of a long acting formulation, computation of a dose
454 using a PK/PD approach requires estimation of the duration of action. To the best of
455 our knowledge, this duration has not been experimentally determined for cattle.
456 However, in pigs duration of effectiveness of tulathromycin was determined in a
457 pulmonary–disease challenge model, using *Actinobacillus pleuropneumoniae* by
458 administering a single dose of tulathromycin from 11 to 3 days before the challenge
459 (Waag, Bradford et al., 2008). These authors concluded that a single dose of
460 tulathromycin provides up to 9 days of protection against death and severe morbidity.
461 For the calves used in this study, we define *a priori* the activity period as that for
462 which the average serum concentration of tulathromycin was lower than the
463 EMA/CVMP BP of 8 µg/mL in MHB (equivalent to 160 ng/mL in serum). The MCSs
464 indicated that the average concentration over the first 10 days following a dose of 2.5
465 mg/kg was 184 ng/mL and this duration was selected to explore the population dose
466 distribution, although any other duration could be investigated using this approach.

467 Computation of a PK/PD dose also requires selection of an appropriate PK/PD index
468 and determination of its BP value. From the rate and extent of killing of *M.*
469 *haemolytica* and *P. multocida* by tulathromycin in time-kill studies, the actions were
470 judged to be, for the former, concentration-dependent in MHB and co-dependent in
471 serum and, for the latter, co-dependent in both matrices (Lees, Illambas et al., 2016).
472 Therefore, for both bacterial species, the use of AUC/MIC as a PK/PD surrogate
473 for/predictor of efficacy is justified (Thomas, Forrest et al., 1998; Schentag, 2000;
474 Evans, 2005). It should be noted that the time-kill studies were conducted with initial
475 inoculum counts of the order of 10^6 to 10^7 cfu/mL *i.e.* for an inoculum size likely to be

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relatively high when considering a natural infection; according to Roof (ROOF, 2011) the total CFU of pathogens in the entire lung for cattle with identified pathogens ranged from $2x10^7$ – $2x10^8$ CFU for *P.multocida* and $9x10^6$ – $9x10^8$ CFU for *M. haemolytica,* indicating that our time-kill curves were indeed obtained with a an appropriate inoculum size, regarding a natural infection. However, for metaphylaxis/control conditions, for which a lower pathogen load is expected, lower concentrations of tulathromycin in the biophase are likely to be required, thus indicating that our results are conservative enough to cover all infectious conditions.

The present findings confirm, in part, previous publications on the PK of tulathromycin (Nowakowski, Inskeep et al., 2004; Evans, 2005). After subcutaneous dosing, a terminal half-life of 90 h was reported by these authors, and in this study half-life was 84 h. However, corresponding $AUC_{(0\text{-last})}$ values were 16.0 µg.h/mL [1,33] and 45.1 µg.h/mL [this study], where $AUC_{(0\text{-last})}$ was measured over similar times. The causes of these differences are unknown. Our data further extends the results of earlier workers, by establishing the distribution of tulathromycin into extravascular fluids (exudate and transudate) in a tissue cage model. This model provides a means of sampling inflammatory exudate to allow comparison of the time course and magnitude of drug penetration into an inflamed (exudate), a non-inflamed (transudate) fluid and in serum (Higgins, Lees et al., 1984; Sidhu, Shojaee Aliabadi et al., 2003). Exudate concentrations were always lower than serum concentration, indicating that there was no specific accumulation of this drug in an inflammatory biophase, as advocated by those assuming a special delivery of tulathromycin in inflammatory fluid by accumulation of macrophages or neutrophils.

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Conclusions

Based on PK/PD modelling, the present study predicts, that the current marketed dose of tulathromycin is appropriate and that serum concentrations achieved with this dosage is biologically and clinically relevant. Recourse to consideration of the high concentration of tulathromycin in lung homogenate or to a specific transport of the drug by leucocytes into the biophase is not necessary, and this conclusion recognizes that the relevant matrix to estimate MIC is serum.

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Conflict of interest statement

P Lees has acted as a consultant to Pfizer Animal Health. He has not consulted ever for Zoetis, only the predecessor company Pfizer on another drug. L. Pelligand has received funding from Zoetis Ltd for non-related studies on maropitant in the dog. None of the other authors has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the contents of the paper.

This does not alter our adherence to PLOS ONE policies on sharing data and materials.

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Table 1. Pharmacokinetic variables for tulathromycin in serum, exudate and transudate in calves

Variable (units)	Serum		Exudate		Transudate	
	Mean	SEM	Mean	SEM	Mean	SEM
C_{max} (μg/mL)	0.75	0.11	0.24	0.016	0.12	0.016
T_{max} (h)*	2.20	1.18	19.7	2.89	18.0	3.69
$T_{½}$ (h)**	84.0	3.61	123.3	10.33	92.1	9.36
$AUC_{(0\text{-}last)}$ (μg.h/mL)	45.1	5.84	18.4	1.99	8.58	0.85
$AUC_{(0\text{-}\infty)}$ (μg.h/mL)	47.9	6.31	20.0	2.24	9.50	0.89
$MRT_{(0\text{-}last)}$ (h)	91.4	2.88	87.2	3.54	83.6	6.39
Cl/F (mL/kg/h)	52.1	7.51	-	-	-	-

Geometric mean unless stated and SEM (n=10) *Arithmetic mean; **Harmonic mean.

T_{max}: Time following dosing at which the maximum concentration (C_{max}) occurred.

$T_{½}$: Terminal Half-life

$AUC_{(0\text{-}last)}$: Area under the concentration-time graph from 0 to the last sampling time

$AUC_{(0\text{-}\infty)}$: Area under the concentration-time graph from 0 to infinity

MRT: Mean residence time

Cl/F: Clearance scaled by bioavailability

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Table 2. Results obtained from the killing curve assay.

Variables	*Mannheimia haemolytica*				*Pasteurella multocida*			
	MHB		Serum		MHB		Serum	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Log E_{max} (cfu/mL)	-4.53	0.66	-5.12	1.43	-5.06	1.41	-4.51	2.03
Log E_0 (cfu/mL)	1.87	0.49	1.97	1.03	1.4	1.04	2.84	1.95
Log Emax-log E_0 (cfu/mL)	-6.40	0.58	-7.09	1.97	-6.46	0.97	-7.37	0.75
Bacteriostatic (AUC_{0-24h}/MICexp)(h)	17.15	7.14	19.75	14.72	18.45	11.59	17.28	9.23
Bactericidal (AUC_{0-24h}/MICexp) (h)	21.04	8.39	38.44	30.35	23.7 (n=5)	18.03	22.72 (n=5)	1.98
$4log_{10}$ reduction in (AUC_{0-24h}/MICexp) (h)	24.86 (n=5)	10.58	32.16 (n=4)	11.6	17.19 (n=4)	9.45	28.83 (n=5)	3.71

Data (mean, SD, n=6 unless stated) for three levels of growth inhibition of *M. haemolytica* and *P. multocida* by tulathromycin in Mueller Hinton broth (MHB) and serum.

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Table 3. Population parameters for tulathromycin disposition in calves.

Parameter	Estimate	SE	CV%
tvKa	10.7	2.75	25.6
tvA	389	123	31.7
tvAlpha	0.2252	0.073	32.4
tvB	365	39	10.6
tvBeta	0.008172	0.00040	4.8
SD of the proportional residual error (eps)	0.245175	0.00683	2.8

Typical values (tv) of the structural parameters of the population model (thetas), their associated Standard Error (SE) and Coefficient of Variation (CV%) and the SD of the residual for the model.

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Table 4. Between Subject Variability (BSV) of tulathromycin disposition in calves.

ETA	ηKa	ηA	ηAlpha	ηB	ηBeta
No covariate	129	344	224	53	8
With the health status as covariate	109	154	122	52	7.8

Coefficient of variation (CV%) of the variance of the etas (η) indicating the magnitude of the BSV for the model without covariate and model with the health status as covariate

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Table 5. PK/PD cut-offs for tulathromycin in calves.

	Intervals (h)									
Percentiles (%)	0-24	0-48	0-72	0-96	0-120	0-144	0-168	0-192	0-216	0-240
95	142	132	120	109	99	91	84	78	72	67
90	**172**	**160**	**145**	**132**	**122**	**111**	**103**	**96**	**89**	**83**
80	210	200	183	167	153	140	129	119	111	104
70	244	229	209	192	176	162	150	139	129	121
60	278	263	242	222	204	188	174	161	150	140
50	316	300	277	255	234	217	201	186	172	161
40	356	337	312	287	264	245	227	210	196	183
30	408	389	360	331	305	283	262	244	228	214
20	476	453	419	384	354	329	305	283	263	245
10	586	555	518	480	445	412	384	359	334	312
5	685	653	611	566	525	488	454	424	396	370

These values are the average serum concentrations (ng/mL) of tulathromycin over 10 incremental time intervals (0-24h, 0-48h….0-240h) that are at least achieved by a given percentage of the calf population (Target Attainment Rate from 5 to 95%) after a single subcutaneous administration of tulathromycin at a dose rate of 2.5 mg/kg. These values should be multiplied by 50 to give the equivalent PK/PD cut-offs in MHB. For example, for 90% TAR and a duration of 0-240 h serum concentration = 4,150 ng/mL and for 50% TAR and a time interval of 0-120 h serum concentration = 11,700 ng/mL.

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Fig.1. Flow chart of the main steps of sample collection, data analysis and results outputs for the PK/PD analysis of tulathromycin in calves.

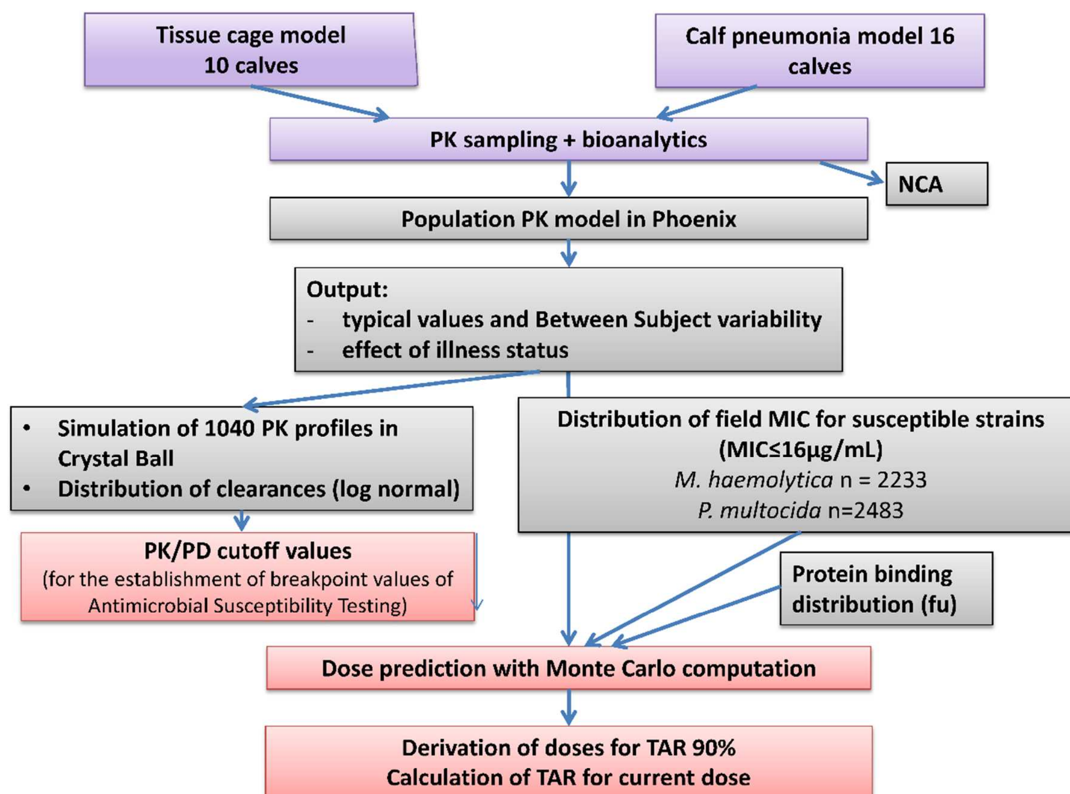

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Fig. 2. Tulathromycin concentration in serum over the first 12 h after subcutaneous administration of tulathromycin in calves. Mean + SEM tulathromycin concentration in serum from 0 to 12 h after subcutaneous injection of tulathromycin at a dose rate of 2.5mg/kg.

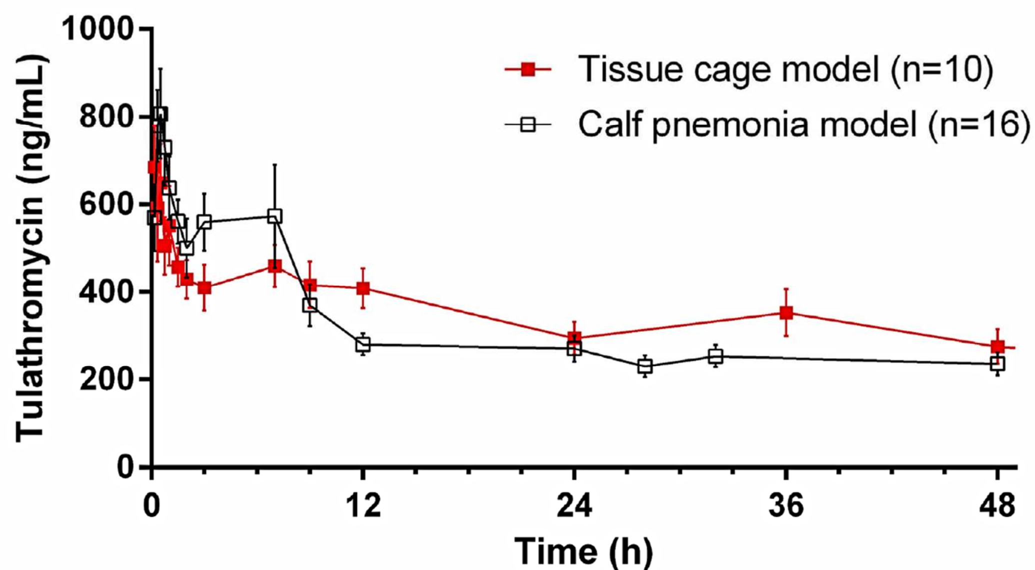

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Fig.3. Tulathromycin concentrations in serum, exudate and transudate over 336 h after subcutaneous administration of tulathromycin in calves. Mean + SEM tulathromycin concentrations in serum, exudate and transudate for calves after subcutaneous injection of tulathromycin at a dose rate of 2.5mg/kg.

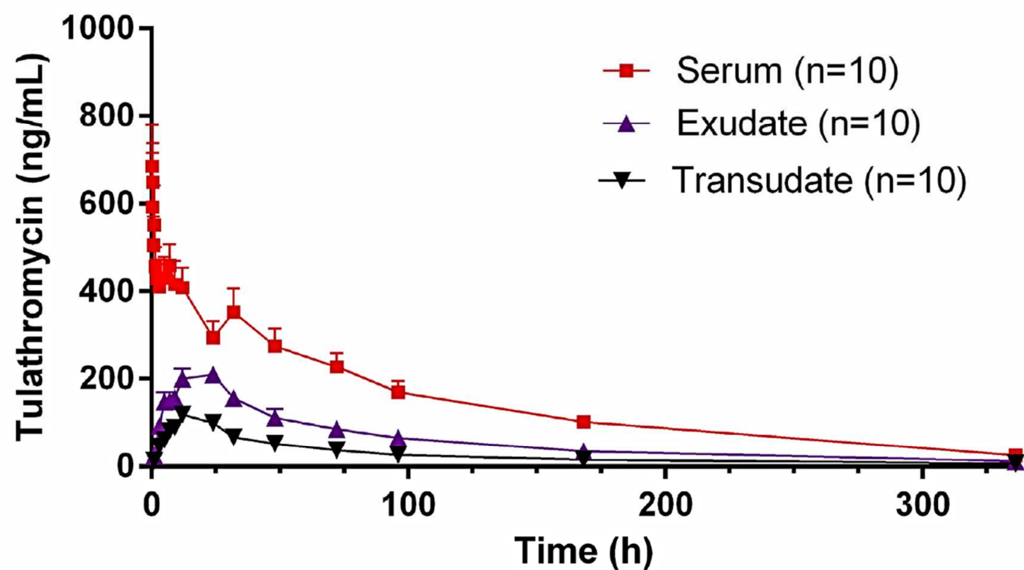

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Fig. 4: Visual Predictive Check: **Observed plasma concentration (ng/ml) vs. time (h) and observed and predicted quantiles.** This diagnostic plot illustrates the observed (red) 5, 50 and 95% quantiles superimposed with the predictive check quantiles (black) 5, 50 and 95% over the observed serum concentrations (blue symbols).

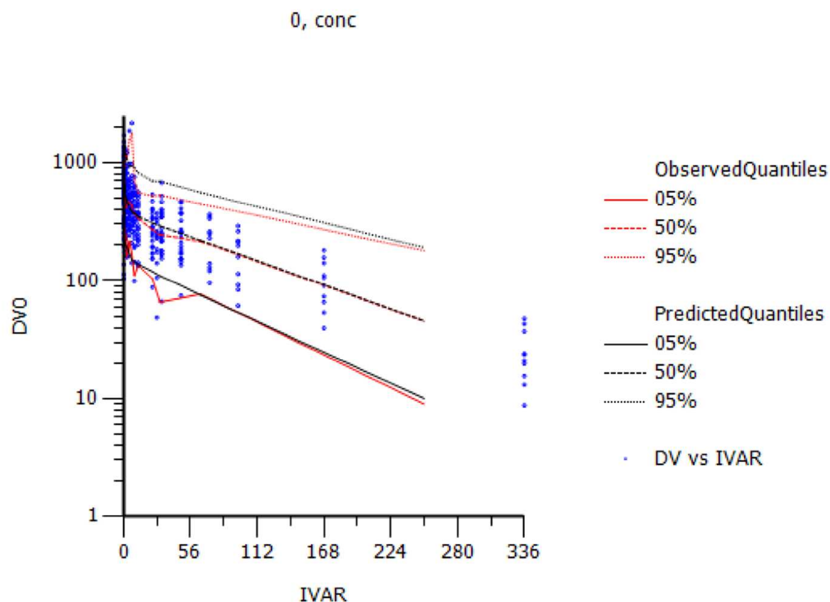

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Fig. 5. MIC distribution of *M. haemolytica* and *P. multocida*. Frequency of Minimum Inhibitory Concentrations (MICs) for tulathromycin against bovine respiratory pathogens *P. multocida* (n=2483) and *M. haemolytica* (n=2233). The isolates were from the Zoetis surveillance program and were isolated from years 2004 to 2010. MICs (µg/mL) were obtained in Mueller Hinton Broth; results are given for only susceptible strains according to the CLSI clinical breakpoint (MICs ≤ 16µg/mL)

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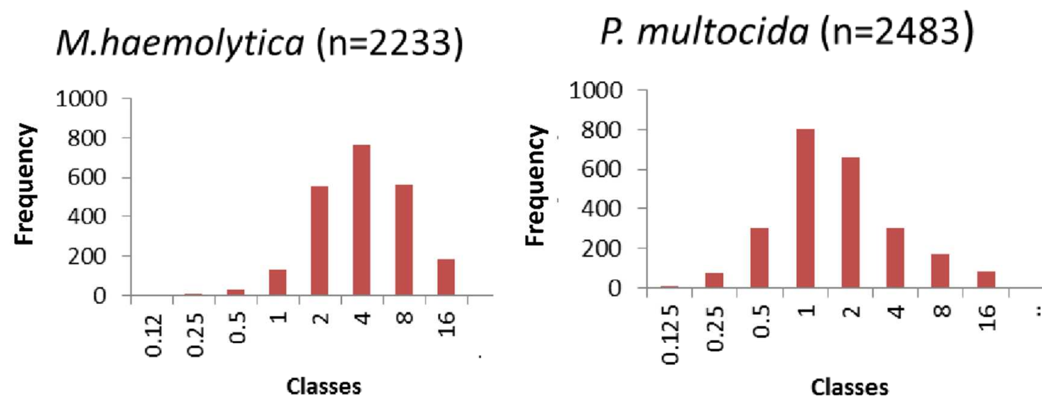

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Fig. 6. Population distributions of tulathromycin doses. Comparison of the two population distributions of tulathromycin doses, as predicted by a population PK/PD model for *P. multocida* (grey) and *M. haemolytica* (blue) for duration of action of 240 h. The vertical bar indicates the nominal dose of 2.5 mg/kg. Dose (0 to 9000 µg/kg) is indicated on the X axis.

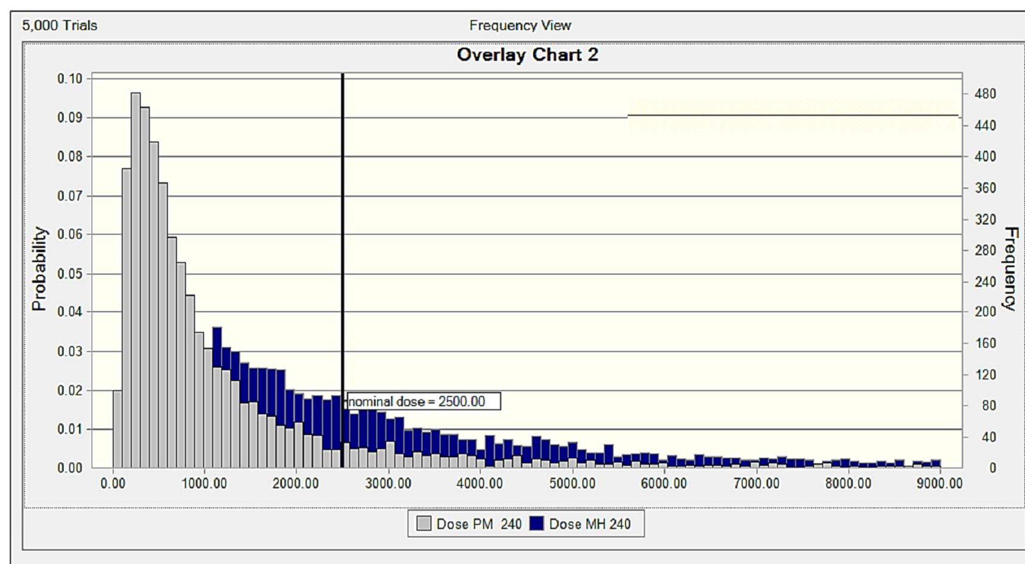

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Fig. 7. Cumulative population distributions of tulathromycin doses. Comparison of the two cumulative distributions of doses of tulathromycin, as predicted by a population PK/PD model for *P. multocida* (grey) and *M. haemolytica* (blue) for a duration of action of 240 h in terms of Target Attainment Rate (Y axis: 0-100%). The vertical bar indicates the nominal dose of 2.5 mg/kg. Dose (0 to 9000 µg/kg) is indicated on the X axis.