Doxycycline serum protein binding in pigs reveals a relatively high free fraction.

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Abstract

Doxycycline is an antibiotic widely used in pig farming, which leads to update the doses using PK/PD concepts. However, there is an impasse to estimate the PK/PD cut-off of doxycycline in pigs. Indeed, considering its 7% free fraction and the total steady state concentrations reported in pig plasma (0.35-1.5 µg/mL), the estimated free concentrations of doxycycline (0.025-0.15 µg/mL) are far lower than the tetracycline MIC_{50} reported for *P. multocida* (MIC_{50} = 0.5 µg/mL). This apparent discrepancy may be explained by the atypical and counter-intuitive non-linear binding of tetracyclines to serum proteins. Plasma protein binding is usually determined by pooling plasma from healthy animals, this does not take into account the inter-subject variability required to define a PK/PD cut-off.

Thus, the protein binding was determined by equilibrium dialysis using individual plasma from twenty-six pigs at doxycycline concentration ranging from 1 to 1000 µM. This study investigated both the shape and measurement of doxycycline protein binding and estimated inter-subject variability using a non-linear mixed effects model.

Our results did not indicate any “atypical” protein binding and reveals a higher free fraction of doxycycline in pig plasma than previously reported (~31%) with a relatively low between-subject variability (~10%).

INTRODUCTION

The tetracycline antimicrobial doxycycline is extensively used in pig farming (Lekagul et al., 2019). Treating pigs with doxycycline at an average in-feed dose of 11 mg/kg/day was shown to effectively control pneumonia caused by *P. multocida* (Bousquet, Pommier, et al., 1998). it was shown that pigs administered medicated feed *ad libitum* at a dose of 11.8-13.3 mg/kg had a steady-state total plasma concentration of doxycycline ranging from 0.9 to 1.5 µg/mL (Bousquet, Nouws, et al., 1998). Other studies predict a somewhat lower value. For example, the average steady-state plasma concentration of doxycycline was predicted to be around 0.35 µg/mL for an in-feed 10 mg/kg oral dose (del Castillo et al., 2006), and around 0.54 µg/mL after administration of doxycycline using a stomach tube (Baert et al., 2000).

These previous studies report the total plasma concentrations of doxycycline, but only free doxycycline has antibacterial activity (Craig & Ebert, 1989) and all PK/PD indices are expressed in terms of free plasma concentration (Toutain et al., 2002, Toutain et al., 2021). Previous work examining serum pooled from six pigs showed that the extent of doxycycline binding to serum protein in pigs was 93.1 +/- 0.25%, and the free fraction was estimated to be about 7% (Riond & Riviere, 1990). More recently, the extent of doxycycline binding to serum proteins was estimated to be 87.8% in healthy pigs and 82.3% in infected pigs (Zhang et al., 2018). Considering the previously reported total plasma concentrations in pigs that were orally administered 10 mg/kg doxycycline, (vide supra), the concentration of free doxycycline in plasma is expected to range from 0.025 to 0.15 µg/mL.

An MIC_{50} of 0.5 µg/mL was reported for tetracycline with isolates of *P. multocida, M. haemolytica , H. somni and B. bronchiseptica* collected from the EU in the period 2009–2012 (El Garch et al., 2016). Similarly, the CLSI Clinical Breakpoint (CBP) for tetracycline in pigs as a class representative of other tetracyclines, is 0.5 µg/mL (Anonymous, 2018). Given that these concentrations are above the predicted concentrations of free doxycycline in plasma in pigs, there has been an impasse in applying PK/PD concepts to estimate the PK/PD cut-off of doxycycline in pigs using Monte Carlo Simulation (MCS). PK/PD cut-off is one of the concentrations (Minimum Inhibitory Concentration, MIC) used to establish a CBP for Antimicrobial Susceptibility Testing (AST) both for CLSI (Watts et al., 2018) and VetCAST (Toutain et al., 2017).

Using AUC/MIC as a PK/PD index and a Probability Target attainment (PTA) of 90%, it was shown from data published by del Castillo et al (del Castillo et al., 2006) that the maximum possible MIC achievable (i.e. the PK/PD cut-off) was 0.25 µg/mL for pigs treated with doxycycline at 10 mg/kg/day (Lees et al., 2006). However, the maximal possible MIC was only 0.025 µg/mL the binding of doxycycline to plasma protein was considered (Lees et al., 2006). Clearly, the PK/PD cut-off estimated using free plasma doxycycline concentrations is about 10-fold lower than the MIC_{50} of clinical isolates. Hence, using standard PK/PD concepts would result in setting very low values for the CBP and rendering doxycycline useless for pigs, at least for the oral route of administration. It was concluded that further studies were required to explain the apparent discrepancy between predictions using PK/PD principles and the results of clinical trials (Lees et al., 2006).

One explanation of this apparent discrepancy may be, in part, the recent discovery that several tetracyclines have an atypical and counter-intuitive non-linear binding to serum protein. The free fraction of several tetracyclines decreases with increasing total plasma concentrations, in sharp contrast to drugs with saturable binding. In humans, the protein binding of tigecycline displays a “U” shaped curve, with free fractions of 34.8%, 7.12%, 3.14% and 21.7% corresponding to total plasma concentrations of 0.1, 1, 10 and 100 µg/mL, respectively (Mukker et al., 2014). In mice, the protein binding of eravacycline also increases non-linearly as total drug concentration increases, with values ranging from 12.5 to 97.3% (Thabit et al., 2016). For doxycycline in mice, the free fraction was approximately 6% (as in pigs) for a concentration of 50 µg/mL but was 5-fold higher, approximately 30%, for a serum concentration of 0.5 µg/mL (Zhou et al., 2017). If doxycycline displays atypical non-linear binding to serum protein in pigs, then the range of free fractions could be higher than expected, potentially explaining the efficacy of doxycycline and its consistency with the PK/PD paradigm.

The first objective of the present study was to revisit doxycycline plasma binding in pigs as historically reported (Riond & Riviere, 1990), given the potentially “atypical” behavior of tetracyclines (Zhou et al., 2017). As with all PK determinants, the extent of protein binding can vary with physiological and pathophysiological covariates. All sources of variability should be investigated when defining a PK/PD cut-off and setting a CBP, but plasma protein binding is generally determined by pooling plasma from healthy animals, which fails to detect inter-subject variability. Indeed, high inter-subject

variability in calves has been reported for the antimicrobials danofloxacin, florfenicol and tulathromycin (Mzyk et al., 2018). Thus, the second objective of our study was to establish doxycycline binding to plasma protein in a relatively large number of individual pigs, and to use a Non-Linear Mixed Effects model to determine their inter-individual variability.

Material and Method

Chemicals

Doxycycline (hyclate form, purity >98%), minocycline hydrochloride (purity >90%), sodium chloride (NaCl), sodium dihydrogenophosphate (NaH2PO4) and disodium hydrogenophosphate (Na2HPO4), trifluoroacetic acid (TFA, purity >99%) and trichloroacetic acid (TCA, purity >99%) were purchased from Merck (Darmstadt, Germany). Methanol (MeOH) was LC/MS quality and purchased from Fisher Scientific (Illkirch, France). Water was obtained from an ultrapure water system (PureLab Classic, Veolia Water).

Plasma sampling

All plasma samples were collected with lithium heparin, centrifuged at 3000 x g for 10 min at 4°C, and stored at -20°C. The first group consisted of six individual piglets used for other animal experiments between 2012 and 2019; they were all 5-10 weeks old and plasma was collected just before euthanasia.

Plasma samples of the second group were collected from ten live male and ten live female piglets, aged of seven weeks of age and 2 months, before the dialysis experiments.

Dialyses to equilibrium of doxycycline

Working solutions of doxycycline were directly prepared in PBS buffer at concentrations ranging from 0.5 µM to 1000 µM. PBS buffer was prepared by diluting 3.19 g of Na2HPO4, 0.67 g of NaH2PO4 and 5.58 g of NaCl in 1 L of ultrapure water. The pH was adjusted to 7.4 and the ionic strength was I = 185. The specific plasma protein binding parameters of doxycycline were evaluated from the plasma of 26 individual piglets (30-60 days old). The equilibrium dialysis system consisted of two Teflon half-cells incubated in a water bath set at 37 °C and under a rotation set at 12

rpm (Diachema 16-10, Braun Scientetec, ZA, Courtaboeuf, Les Ulis, France). Doxycycline solution at 10; 50; 100; 500; and 1000 µM in PBS (900 µL) was incubated with pig plasma (900 µL) for 14 hours at 37 °C, separated by a dialysis membrane with a cut-off of 12-14000 Daltons (Medicell Membranes Ltd, London, UK). Each compartment (buffer and plasma) was collected and assayed by high performance liquid chromatography (HPLC) with UV detection.

Tetracycline assays

Doxycycline plasma and buffer samples (100 µL) were extracted from the matrix by adding 10 µL of IS (Minocycline 50 µg/mL) and 200 µL of 5% TCA aqueous solution. The mixture was shaken at 10 °C for 2 min at 1400 rpm (MB-102, Bioer, Hangzhou, China) and centrifuged for 10 min at 4°C and 20000 x g. Samples were analyzed by ultra-performance liquid chromatography with a diode array UV detection (Acquity UPLC®, Waters, Milford, MA, USA). Doxycycline and minocycline were separated on a C18 column (Acquity 50 x 2.1 mm, 1.7 µm, Waters) using an ACN 0.1% TFA /H_2O 0.1% TFA gradient elution at 0.4 ml/min flow rate and detected at λabs = 350 nm. The linear gradient of the mobile phase was as follows: (0 min, 90% H_2O; 0.5 min 90% H_2O, 2.5 min 10% H_2O; 2.7 min 90% H_2O, 4.5 min 90% H_2O). Doxycycline concentrations were determined using a linear model weighted by $1/X^2$ (X= nominal concentration). The calibration curves ranged from 0.5-1000 µM and 1-1000 µM for doxycycline determination in PBS and in plasma, respectively. Each calibration curve was validated with at least 4 QC samples and the RCR (relative concentration residuals) were systematically lower than 11%. Doxycycline was stable for at least 14 hours in both PBS and in plasma (RCR % lower than 12%).

Data analysis

Data expressed in molar concentrations were analyzed using a Non-linear Mixed Effect modelling approach (NLME) in Phoenix (Phoenix NLME version 8.3, Certara, St. Louis MO, United States). The empirical structural model developed to describe the atypical binding of tigecycline in human serum (Singh et al., 2017) was used (Eq.1):

$$fu = Beta * TOT^{Alpha} \qquad \text{Eq.1}$$

Where fu is the measured unbound fraction (from 0 to 1), TOT is the measured total plasma doxycycline concentration (µmol/L), Beta is the typical value of fu when

Alpha=0, Alpha is an exponent whose value allows fu to be different depending on the level of the total doxycycline plasma concentrations. If Alpha is equal to 0, fu is constant and equal to Beta over the whole range of total doxycycline plasma concentrations. If Alpha is different from 0 then fu is non-linear, and the degree of non-linearity correlates with the difference of Alpha from 0. When Alpha is negative (the case of atypical binding), fu decreases with the increase in plasma concentrations, whereas if Alpha is positive, an increase in the free fraction is observed with the increase in total plasma concentrations, which reflects the classic case of a saturable plasma binding.

The Between Subject Variability (BSV) for Beta and Alpha estimated by the variance across individuals was modeled using an exponential model of the form:

$$\theta_{Parameters\_i} = \theta_{tv\_Pamameters} \times Exp(\eta_i) \quad \text{Eq: 2}$$

With $\theta_{Parameters\_i}$ is the value of Beta or Alpha in the ith pig, $\theta_{tv\_Parameters}$ is the typical population value of Beta or Alpha (see equation 2) and η_i, the deviation associated to the *ith* pig from the corresponding population value. When parameters are treated as arising from a log-normal distribution, the variance estimate (ω^2) is the variance in the log-domain, and this was converted to a coefficient of variation (CV%) in the original scale with Eq:4:

$$\mathrm{CV}(\%) = 100 \times \sqrt{\exp(\omega^2) - 1} \quad \text{Eq: 3}$$

Shrinkage of random effects toward the means could have occurred due to the rather sparse sampling, and a metric for shrinkage was calculated for the etas and epsilon (Karlsson & Savic, 2007). When shrinkage for eta are > 30%, it was considered that data were not rich enough to robustly estimate this random component from the model. The shrinkage for the etas was estimated as follows:

$$shrinkage = 1 - \frac{SD(EBE_\eta)}{\omega} \quad \text{Eq: 4}$$

where ω is the estimated variability for the population and SD is the SD of the individual values of the Empirical Bayesian Estimates (EBE) of η.

The residual model was an additive plus a multiplicative (proportional) model. The additive sigma is reported as its standard deviation noted *stdev*, with the same units as plasma concentration (µmol/L) and the multiplicative sigma is the corresponding coefficient of variation.

The free plasma doxycycline concentration corresponding to a given total plasma concentration can be computed from the model with equation 5:

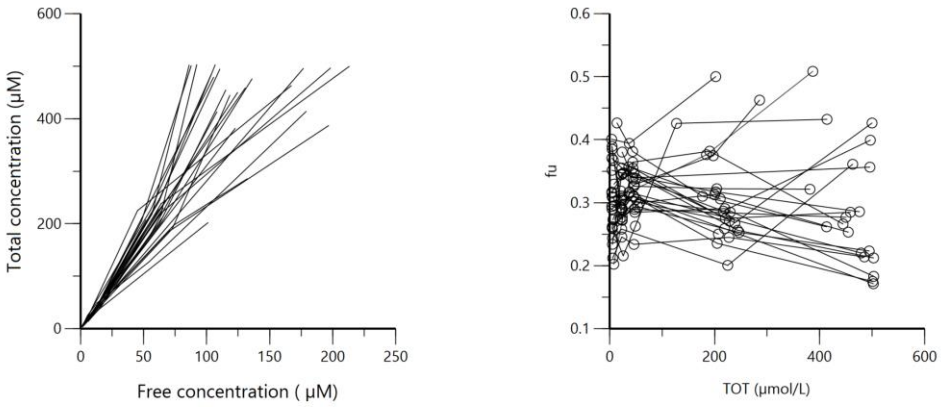
$$Free = Beta * TOT^{(Alpha+1)} \qquad \text{Eq: 5}$$

Parameter estimation was based on minimizing an objective function value, using maximum likelihood estimation by a Laplacian engine. Precision of parameters were estimated using the Phoenix bootstrap tool.

Results

The individual curves showing the relationship between free and total concentrations and between fu and the total plasma doxycycline concentrations for 26 pigs are depicted on figure 1.

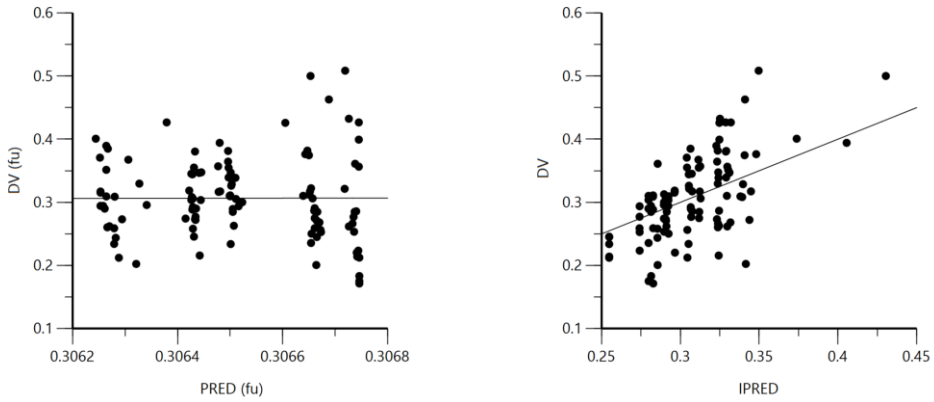
Figure 1 Spaghetti plot of individual curves showing the relationship between free (X-axis) and total plasma doxycycline concentration (Y-axis) (left) and between total plasma doxycycline concentration (X-axis) and fu, the free fraction (Y-axis) (right) in 26 pigs.



Visual inspection of the data in figure 1 did not indicate an “atypical” protein binding (no “U” curve) but a rather linear relationship between free and total concentrations of doxycycline.

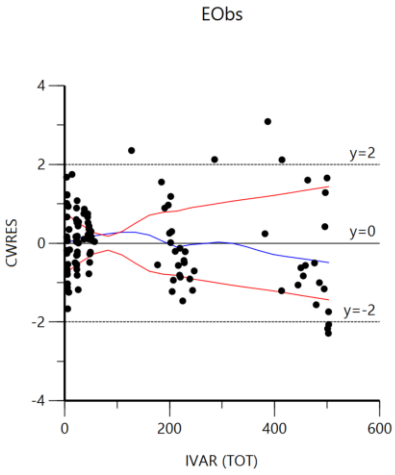
Figures 2 to 4 are Goodness-of-fit (GOF) plots supporting the structural models, the exponential model for the random component on Alpha and Beta, and the additive plus multiplicative error model that were used to analyze the data.

Figure 2: DV vs PRED (left) and DV vs IPRED (right). Plot of the dependent variable (DV) i.e. of fu versus population predicted fu (PRED) (no random component) or IPRED obtained by setting random effects to the 'post hoc' or empirical Bayesian estimate of the random effects i.e. for each individual Beta and Alpha .



For both plots, the data were evenly distributed around the line of identity, indicating no major bias in the population component of the model.

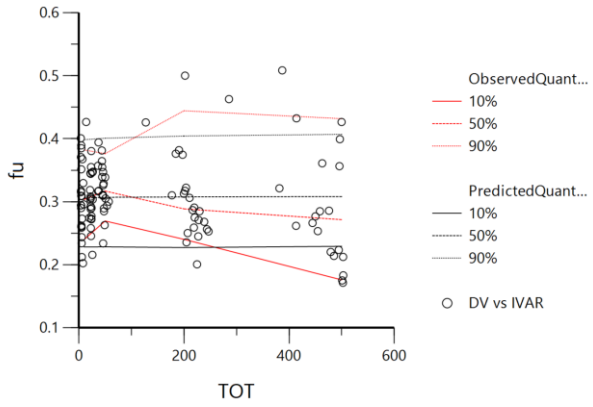
Figure 3 CWRES vs. total plasma concentration. Plot of CWRES (conditional weighted residuals), goodness of fit statistic, against the independent variable (Total doxycycline plasma concentration). Values of CWRES should be approximately N(0,1) and hence concentrated between y=-2 and y=+2. Values significantly above 3 or below -3 are suspect and may indicate a lack of fit and/or model misspecification.



Inspection of figure 3 shows that data are evenly distributed around zero (see the trends as given by the blue line), indicating no major bias in the structural model and the error model. Ideally, the blue line should be at 0 and the red line (with its negative reflection) should not show any fanning.

The overall adequacy of the model was assessed by plotting the Visual Predictive Check **(**VPC). Using the identified model, 1000 replications based on the structure of the original data were simulated and the distribution of these replicates were compared to actual observations. The 10th, 50th and 90th quantile of the simulated distribution were compared to the observations.

Figure 4 Visual Predictive Check (VPC) obtained with 1000 replicates of each animal. Red line: observed quantiles; Black lines: predicted quantiles; Black symbols: observed data. The observed quantiles (10, 50 and 90%) were reasonably superimposed with the corresponding predictive check quantiles over the observed data.



Typical values of Alpha, Beta, value of variance of the BSV for Alpha and Beta and the corresponding BSV in % and multiplicative and additive components of the residual error are given in table 1.

Table 1: Thetas: Population primary parameters (Beta, Alpha); Omega gives the variance of the Between Subject Variability (BSV) and BSV% as a coefficient of variation (CV%) of the primary parameters. Correlation is the correlation between Beta and Alpha estimates. Bootstrap estimates of precision are given by a (CV%), 2.5% and 97.5% percentiles for thetas and for the two components of residual error and by SE for Omega and correlation.

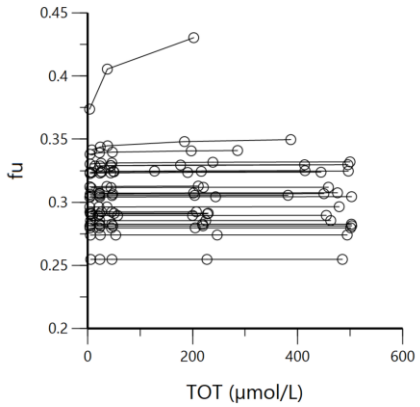
Thetas (Parameters)	Estimate	Units	CV%	2.5% CI	97.5% CI
tvBeta	0.306	scalar	1.28	0.294	0.308
tvAlpha	0.00033	scalar	7.46	0.000237	0.000342
tvMultStdev	0.180	scalar	7.18	0.142	0.190
stdev0	0.01000	µmol/L	3.15	0.00926	0.0100
OMEGA			SE		
nBeta	0.011		0.00296		
nAlpha	0.149	5.571	0.0209		
BSV%	10.32	1617			
Shrinkage	0.261	0.379			
Correlation					
nBeta	1				
nAlpha	0.614	1	0.741		

Tv: typical value; Multiplicative component of the error model is expressed as CV% and the additive component of the residual error model by its standard deviation (Stdv).

Inspection of table 1 indicates that the typical value of Beta is 0.306 and that Alpha was very small and biologically irrelevant even if statistically significant. Hence, the typical value of fu was estimated to be 0.306.

Figure 5 depicts the relationship between the unbound fraction (fu) and the total measured doxycycline concentrations (µmol/L) in 26 pigs as given by the post hoc estimate of individual Beta and Alpha.

Figure 5: unbound fraction (fu) vs. total measured doxycycline concentrations (µmol/L) in 26 pigs as given by the post hoc estimate of individual Beta and alpha .



For all but one pig, the unbound fraction was practically constant with an average value of 30.6 % in the range of therapeutic plasma concentration (from 0.1 to 10 µg/mL i.e. from 0.225 to 22.5 µmol/L) .

From the post hoc estimates of Beta and Alpha of the 26 pigs, fu was estimated to 30.6 +/-2.47% (mean and SD) for a total plasma concentration of 1.0 µg/mL.

Discussion

Here, we examined doxycycline binding to plasma protein in 26 individual pigs, and use a Non-Linear Mixed Effects model to determine their inter-individual variability. Our data did not reveal any atypical binding of doxycycline in pig serum, in contrast with observations with doxycycline in human and mouse serum (Zhou et al., 2017) and other tetracyclines, such as tigecycline, in human serum (Singh et al., 2017). We found that the binding of doxycycline in pig serum was relatively lower than previously reported (fu=31%), linear and without evidence of saturation over the large range of plasma concentrations tested and covering all those studied previously in pigs (0.375 -300 µg/mL) (Riond & Riviere, 1989 & 1990; Zhang et al., 2018).

The free fraction of doxycycline in pig plasma that we detected (31%) is higher than previously reported values of 7% (Riond & Riviere, 1990) and 12% (Zhang et al., 2018).

The origin(s) of this difference remains unclear, but could reflect methodological aspects and/or of biological variability in pig populations. The value reported by Riond and Riviere was determined from a single doxycycline concentration of 10 µg/mL (22.5 µmol/L) and with pooled serum (Riond & Riviere, 1990). Zhang et al. also used pooled serum, but binding was tested at lower concentrations of doxycycline (1.5, 0.75, and 0.375 µg/mL or 0.84, 1.69 and 3.37 µmol/L) using ultrafiltration (Zhang et al., 2018), a method that is not without difficulties (Metsu et al., 2020). At present, it is not clear whether a pooled serum can provide an authentic mean value of the protein binding or whether it suffers from a potential bias associated with Naïve Pooling Data, when averaging unbalanced data (Hing et al., 2001). In our assay, we measured the extent of individual plasma protein binding in plasma samples from 26 different pigs, sweeping a large range of semi-logarithmically spaced total doxycycline concentrations to assess finely a potential non-linearity.

The second objective of our study was to estimate the BSV of protein binding, a source of variability rarely taken into account when discussing PK/PD relationship for antimicrobials. This source of variability cannot be ignored when attempting to estimate the concentration of free doxycycline from the concentration of total doxycycline in plasma. However, a variability in fu is not equivalent to a variability in the corresponding free (microbiologically active) concentration of doxycycline in plasma in vivo. The free plasma concentration is controlled only by the free plasma clearance, not the extent of protein binding (Toutain & Bousquet-Melou, 2002). An alteration or a variability of fu, which is a hybrid variable (fu=Cfree/CTot), may simply reflect a variability of the total plasma doxycycline concentration that can be explained by a variability of the albumin concentration or by its affinity for doxycycline, which would not affect the concentration of free doxycycline.

Another motivation for our study was to determine if it was possible to compute a realistic PK/PD cut-off of doxycycline in pigs, and thus establish CBPs. With a binding of 7%, a very low PK/PD cut-off of 0.025 µg/mL was historically computed for an oral dose of 10 mg/Kg (Lees et al., 2006). With a 4-fold higher fu, the PK/PD cut-off would be about 0.1 µg/mL with an oral dose of 10 mg/kg or 0.2 µg/mL for an oral dose of 20 mg/kg as is currently recommended. The MIC_{50} of tetracyclines has been reported to be 0.50 µg/mL for the main pathogens in pigs (El Garch et al., 2016). Comparing the MICs of doxycycline and oxytetracycline on the same strains of *P multocida* and *A*

pleuropneumoniae revealed that doxycycline was more potent than oxytetracycline for susceptible strains (Bousquet et al., 1997). Similar results were obtained in a comparative study of the potency of five tetracyclines (Pijpers et al., 1989). The ratio of tetracycline/doxycycline potency was approximatively 2-4; thus, the MIC_{50} of 0.50 µg/mL reported for tetracycline in pigs could be equivalent to 0.125-0.250 µg/mL doxycycline. Additionally, a potential matrix effect on the doxycycline potency should be considered. Similar killing curves have been obtained in pig serum (ex-vivo experiment) and in tryptic soy broth as the culture medium, despite doxycycline binding to plasma proteins in serum by 87% (Zhang et al., 2018). In this study, critical values of the PK/PD index were computed from total (not free) concentrations of doxycycline, which does not consider doxycycline binding to plasma proteins (Zhang et al., 2018). Indeed, a matrix effect was reported for pig serum when testing oxytetracycline against *A pleuropneumoniae*, with a potency about 3-fold higher in serum than in tryptic soy broth or Mueller Hinton Broth after correcting for binding to plasma proteins (Dorey & Lees, 2017).

Conclusions

Our study reveals a higher free fraction of doxycycline in pig plasma than previously reported, facilitating the calculation of realistic and useful CBPs for doxycycline in pigs.

FUNDING

This study was financed with own's laboratory funds.

CONFLICT OF INTEREST

All Authors declare that they have no competing interests.

DATA SHARING AND DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ANIMAL WELFARE AND ETHICS STATEMENT;

The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (as adopted and promulgated by the U.S. National Institutes of Health and) under the agreement numbers #24958-2020032317345863 and

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AUTHOR CONTRIBUTIONS

P-L Toutain, V Gayrard and MZ Lacroix conceived and planned the experiments. F Ramon-Portugal, BB Roques and MZ Lacroix contributed to sample collection and carried out the experiments. P-L Toutain and A Bousquet-Melou carried out the simulations. P-L Toutain, A Bousquet-Melou, V Gayrard and MZ Lacroix contributed to the interpretation of the results. P-L Toutain drafted the first version of this article. All authors edited subsequent versions of the article.

REFERENCES

Anonymous. (2018). CLSI.Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals. 4th ed. CLSI supplement VET08. Wayne, PA: Clinical and Laboratory Standards Institute;

Baert, K., Croubels, S., Gasthuys, F., De Busser, J., & De Backer, P. (2000). Pharmacokinetics and oral bioavailability of a doxycycline formulation (DOXYCYCLINE 75%) in nonfasted young pigs. Journal of Veterinary Pharmacology and Therapeutics, 23(1), 45–48.

Bousquet, E., Morvan, H., Aitken, I., & Morgan, J. H. (1997). Comparative in vitro activity of doxycycline and oxytetracycline against porcine respiratory pathogens. The Veterinary Record, 141(2), 37–40. https://doi.org/10.1136/vr.141.2.37

Bousquet, E., Nouws, J., Terlouw, P., & de Kleyne, S. (1998). Pharmacokinetics of doxycycline in pigs following oral administration in feed. Veterinary Research, 29(5), 475–485.

Bousquet, E., Pommier, P., Wessel-Robert, S., Morvan, H., Benoit-Valiergue, H., & Laval, A. (1998). Efficacy of doxycycline in feed for the control of pneumonia caused by Pasteurella multocida and Mycoplasma hyopneumoniae in fattening pigs. The Veterinary Record, 143(10), 269–272.

Craig, W. A., & Ebert, S. C. (1989). Protein binding and its significance in antibacterial therapy. Infectious Disease Clinics of North America, 3(3), 407–414.

del Castillo, J. R. E., Laroute, V., Pommier, P., Zémirline, C., Keïta, A., Concordet, D., & Toutain, P.-L. (2006). Interindividual variability in plasma concentrations after systemic exposure of swine to dietary doxycycline supplied with and without paracetamol: A population pharmacokinetic approach1. Journal of Animal Science, 84(11), 3155–3166. https://doi.org/10.2527/jas.2005-561

Dorey, L., & Lees, P. (2017). Impact of growth matrix on pharmacodynamics of antimicrobial drugs for pig pneumonia pathogens. BMC Veterinary Research, 13(1), 192. https://doi.org/10.1186/s12917-017-1086-4

El Garch, F., de Jong, A., Simjee, S., Moyaert, H., Klein, U., Ludwig, C., Marion, H., Haag-Diergarten, S., Richard-Mazet, A., Thomas, V., & Siegwart, E. (2016). Monitoring of antimicrobial susceptibility of respiratory tract pathogens isolated from diseased cattle and pigs across Europe, 2009–2012: VetPath results. Veterinary Microbiology, 194, 11–22. https://doi.org/10.1016/j.vetmic.2016.04.009

Hing, J. P., Woolfrey, S. G., Greenslade, D., & Wright, P. M. (2001). Is mixed effects modeling or naïve pooled data analysis preferred for the interpretation of single sample per subject toxicokinetic data? Journal of Pharmacokinetics and

Pharmacodynamics, 28(2), 193–210. https://doi.org/10.1023/a:1011507100493

Karlsson, M. O., & Savic, R. M. (2007). Diagnosing model diagnostics. Clinical Pharmacology and Therapeutics, 82(1), 17–20. https://doi.org/10.1038/sj.clpt.6100241

Lees, P., Concordet, D., Aliabadi, F. S., & Toutain, P. L. (2006). Drug selection and optimization of dosage schedules to minimize antimicrobial resistance. In Antimicrobial resistance in bacteria of animal origin. Aarestrup FM (editor). ASM Press.

Lekagul, A., Tangcharoensathien, V., & Yeung, S. (2019). Patterns of antibiotic use in global pig production: A systematic review. Veterinary and Animal Science, 7, 100058. https://doi.org/10.1016/j.vas.2019.100058

Metsu, D., Lanot, T., Fraissinet, F., Concordet, D., Gayrard, V., Averseng, M., Ressault, A., Martin-Blondel, G., Levade, T., Février, F., Chatelut, E., Delobel, P., & Gandia, P. (2020). Comparing ultrafiltration and equilibrium dialysis to measure unbound plasma dolutegravir concentrations based on a design of experiment approach. Scientific Reports, 10(1), 12265. https://doi.org/10.1038/s41598-020-69102-y

Mukker, J. K., Singh, R. P., & Derendorf, H. (2014). Determination of atypical nonlinear plasma−protein-binding behavior of tigecycline using an in vitro microdialysis technique. Journal of Pharmaceutical Sciences, 103(3), 1013–1019. https://doi.org/10.1002/jps.23872

Mzyk, D. A., Bublitz, C. M., Hobgood, G. D., Martinez, M. N., Davis, J. L., Smith, G. W., & Baynes, R. E. (2018). Effect of age on plasma protein binding of several

veterinary drugs in dairy calves 2. Research in Veterinary Science, 121, 59–64. https://doi.org/10.1016/j.rvsc.2018.09.004

Pijpers, A., Van Klingeren, B., Schoevers, E. J., Verheijden, J. H., & Van Miert, A. S. (1989). In vitro activity of five tetracyclines and some other antimicrobial agents against four porcine respiratory tract pathogens. Journal of Veterinary Pharmacology and Therapeutics, 12(3), 267–276. https://doi.org/10.1111/j.1365-2885.1989.tb00670.x

Riond, J. L., & Riviere, J. E. (1989). Doxycycline binding to plasma albumin of several species. Journal of Veterinary Pharmacology and Therapeutics, 12(3), 253–260.

Riond, J. L., & Riviere, J. E. (1990). Pharmacokinetics and metabolic inertness of doxycycline in young pigs. American Journal of Veterinary Research, 51(8), 1271–1275.

Singh, R. S. P., Mukker, J. K., Drescher, S. K., Deitchman, A. N., & Derendorf, H. (2017). A need to revisit clinical breakpoints of tigecycline: Effect of atypical non-linear plasma protein binding. International Journal of Antimicrobial Agents, 49(4), 449–455. https://doi.org/10.1016/j.ijantimicag.2016.12.008

Thabit, A. K., Monogue, M. L., & Nicolau, D. P. (2016). Eravacycline Pharmacokinetics and Challenges in Defining Humanized Exposure In Vivo. Antimicrobial Agents and Chemotherapy, 60(8), 5072–5075. https://doi.org/10.1128/AAC.00240-16

Toutain, P. L., & Bousquet-Melou, A. (2002). Free drug fraction vs free drug concentration: A matter of frequent confusion. Journal of Veterinary Pharmacology and Therapeutics, 25(6), 460–463.

Toutain, P., Pelligand, L., Lees, P., Bousquet-Mélou, A., Ferran, A. A., & Turnidge, J. D. (2021). The pharmacokinetic/pharmacodynamic paradigm for antimicrobial drugs in veterinary medicine: Recent advances and critical appraisal. Journal of Veterinary Pharmacology and Therapeutics, 44(2), 172–200. https://doi.org/10.1111/jvp.12917

Toutain, P.-L., Bousquet-Mélou, A., Damborg, P., Ferran, A. A., Mevius, D., Pelligand, L., Veldman, K. T., & Lees, P. (2017). En Route towards European Clinical Breakpoints for Veterinary Antimicrobial Susceptibility Testing: A Position Paper Explaining the VetCAST Approach. Frontiers in Microbiology, 8, 2344. https://doi.org/10.3389/fmicb.2017.02344

Toutain, P.-L., del Castillo, J. R. E., & A Bousquet-Mélou. (2002). The pharmacokinetic–pharmacodynamic approach to a rational dosage regimen for antibiotics. Research in Veterinary Science, 73(2), 105–114. https://doi.org/10.1016/S0034-5288(02)00039-5

Watts, J. L., Sweeney, M. T., & Lubbers, B. V. (2018). Antimicrobial Susceptibility Testing of Bacteria of Veterinary Origin. Microbiology Spectrum, 6(2). https://doi.org/10.1128/microbiolspec.ARBA-0001-2017

Zhang, L., Li, Y., Wang, Y., Sajid, A., Ahmed, S., & Li, X. (2018). Integration of pharmacokinetic-pharmacodynamic for dose optimization of doxycycline against Haemophilus parasuis in pigs. Journal of Veterinary Pharmacology and Therapeutics, 41(5), 706–718. https://doi.org/10.1111/jvp.12512

Zhou, J., Tran, B. T., & Tam, V. H. (2017). The complexity of minocycline serum protein binding. Journal of Antimicrobial Chemotherapy, 72(6), 1632–1634. https://doi.org/10.1093/jac/dkx039