**Research in Veterinary Science**

# Potency of marbofloxacin for pig pneumonia pathogens *Actinobacillus pleuropneumoniae* and *Pasteurella multocida*: comparison of growth media

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Short title: Marbofloxacin and pig pathogens

## Abstract

Pharmacodynamic properties of marbofloxacin were established for six isolates each of the pig respiratory tract pathogens, *Actinobacillus pleuropneumoniae* and *Pasteurella multocida.* Three *in vitro* indices of potency were determined; Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Mutant Prevention Concentration (MPC). For MIC determination Clinical Laboratory Standards Institute guidelines were modified in three respects: (1) comparison was made between two growth media, an artificial broth and pig serum; (2) a high inoculum count was used to simulate heavy clinical bacteriological loads; and (3) five overlapping sets of two-fold dilutions were used to improve accuracy of determinations. Similar methods were used for MBC and MPC estimations. MIC and MPC serum:broth ratios for *A. pleuropneumoniae* were 0.79:1 and 0.99:1, respectively, and corresponding values for *P. multocida* were 1.12:1 and 1.32:1. Serum protein binding of marbofloxacin was 49%, so that fraction unbound (fu) serum MIC values were significantly lower than those predicted by correction for protein binding; fu serum:broth MIC ratios were 0.40:1 (*A. pleuropneumoniae*) and 0.50:1 (*P. multocida*). For broth, MPC:MIC ratios were 13.7:1 (*A. pleuropneumoniae*) and 14.2:1 (*P. multocida*). Corresponding ratios for serum were similar, 17.2:1 and 18.8:1, respectively. It is suggested that, for dose prediction purposes, serum data might be preferable to potency indices measured in broths.



**Keywords:**

Marbofloxacin; Minimum Inhibitory Concentration; Minimum Bactericidal Concentration; Mutant Prevention Concentration; *Pasteurella multocida; Actinobacillus pleuropneumoniae*

## 1. Introduction

Internationally accepted methods, guidelines and standards for Minimum Inhibitory Concentration (MIC) determination have been set by the European Union Committee on Antimicrobial Sensitivity testing (EUCAST) and the Clinical Laboratory Standards Institute (CLSI). These standards provide the advantage of consistency in comparing MICs between individual experimenters, laboratories and across countries (Papich 2013). For MIC determination, EUCAST and CLSI require the use of two-fold dilutions. When plotted on a histogram, using a log-base 2 distribution, the distributions are log-normal. These histograms facilitate the identification of wild-type distributions. CLSI reports microbiological Cut-Offs ($CO_{WT}$) and EUCAST reports Epidemiological Cut-Offs (ECOFF). These are often identical but differences occur for some drugs.

Despite these clear benefits, for the purposes of the present study, the CLSI/EUCAST methods of determining MIC have two disadvantages. First, being based on two-fold dilutions, there is potential for up to 100% error on single isolate estimates, thus having a limitation regarding accuracy for a small number of isolates. To partially meet this concern, previously we have used five sets of overlapping two-fold dilutions; this reduces inaccuracy from approaching 100% to not exceeding 20% (Aliabadi and Lees, 2001; Sidhu et al., 2010). Second, the CLSI/EUCAST standards are based on the use of broths, specifically formulated to facilitate bacterial growth *in vitro*. They differ in composition from biological fluids and hence may not reflect bacterial growth conditions *in vivo.* To enable comparisons between broths and biological fluids as growth matrices, and to evaluate possible differences between them, previous authors have used serum, plasma and inflammatory exudate (Aliabadi and

Lees, 2001, 2002; Nightingale and Murakawa, 2002; Zeitlinger et al., 2004, 2008; Sidhu et al., 2010).

To optimise clinical efficacy and minimise the emergence of resistance to antimicrobial drugs, a third consideration is dosage required for differing pathogen loads. For metaphylaxis and treatment early in the course of disease, when the pathogen load is absent or low, many drugs will either prevent or cure disease, acting in support of natural body defences. However, this general consideration does not apply to marbofloxacin, which is recommended solely for therapeutic use. The major challenge for antimicrobial drugs is to select a dosage regimen which provides a bacteriological cure and avoids the emergence of resistance, when pathogen numbers in the biophase are high (Mouton et al., 2011a, 2011b; Martinez et al., 2012; Papich, 2014). For this reason, a high starting inoculum count of approximately $10^7$CFU/mL was selected for use in this study, in preference to the inoculum count of $5x10^5$CFU/mL recommended in CLSI and EUCAST guidelines.

The product literature for marbofloxacin contains the statement, "Official and local antimicrobial policies should be taken into account when the product is used. Fluoroquinolones should be reserved for the treatment of clinical conditions which have responded poorly, or are expected to respond poorly, to other classes of antimicrobials. Whenever possible, fluoroquinolones should only be used based on susceptibility testing. Use of the product deviating from the instructions given in the SPC/datasheet may increase the prevalence of bacteria resistant to the fluoroquinolones and may decrease the effectiveness of treatment with other quinolones due to the potential for cross resistance."

The aims of this investigation were: (1) to determine the degree of protein binding of marbofloxacin in pig serum; (2) for marbofloxacin and six isolates each of two pig respiratory pathogens (*A. pleuropneumoniae* and *P. multocida*) to determine three indices of

potency, MIC, Minimum Bactericidal Concentration (MBC) and Mutant Prevention Concentration (MPC) using five sets of overlapping two-fold dilutions and to compare each index in two matrices, CLSI recommended broths and pig serum.

## 2. Materials and Methods

*2.1 Marbofloxacin serum protein binding*

Marbofloxacin concentration in pig serum *in vitro* was determined by high pressure liquid chromatography (HPLC) (Aliabadi and Lees, 2002). The HPLC system comprised a Dionex Ultimate 3000 pump and autosampler connected to a Dionex RF 2000 fluorescence detector (Thermo Fisher UK Ltd., Hemel Hempstead, UK). Fluorescence detection was set at an excitation wavelength of 295 nm and an emission wavelength of 500 nm. Chromatographic data were analysed using Chromeleon and concentrations of marbofloxacin were calculated using ratios of peak area marbofloxacin:internal standard. Marbofloxacin concentrations used were 0, 0.0025, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 µg/mL, incubated for 30 min. Two sample sets were used: (1) spiked marbofloxacin standards in serum to determine total concentration; (2) an aliquot of these serum samples filtered using ultra-filtration devices (Amicon Ultra Centrifugal filters, Ultracel 10k, Sigma-Aldrich Ltd., Dorset, UK). The 3 mL sample aliquot was placed in the ultra-filter unit and centrifuged at 4000xg for 20 min at 25$^{o}$C. The ultra-filtrate was harvested from the reservoir of the system and assayed to determine concentration in the protein free fraction. For each concentration, determinations were made on three batches of pig serum.

$$\% \text{ Protein Binding} = \frac{[\text{total} - \text{unbound}] \quad x100}{\text{Total}}$$

*2.2 Bacterial isolates*

Twenty isolates of *P. multocida* were supplied by Don Whitley Scientific (Shipley, West Yorkshire, UK). They also supplied three ATCC reference strains for use in MIC tests; *A. pleuropneumoniae* ATCC 27090, *Enterococcus faecalis* ATCC 29212 and *E. coli* ATCC 25922. Eight isolates of *A. pleuropneumoniae* were supplied by A. Rycroft (Royal Veterinary College, Herts., UK). All *A. pleuropneumoniae* and *P. multocida* isolates were derived from EU field cases of pig pneumonia. They were stored at -80$^{o}$C in 10% Marvel® milk powder, 15% glycerol in sterile distilled water. The mixture was sterilized by boiling for 5 sec, left to cool for 12 h and then boiled again for a further 5 sec.

Six isolates of each species were selected, based on three criteria: (1) ability to grow logarithmically in both CLSI recommended broth and pig serum; (2) susceptibility to marbofloxacin, as indicated by MIC determined using doubling dilutions; and (3) selection of isolates with the highest and lowest broth MICs plus four isolates with intermediate MICs.

*2.3 Culture methods and bacterial counts*

For *A. pleuropneumoniae,* Chocolate Mueller Hinton Agar (CMHA) was used for growth on a solid medium and Columbia broth supplemented with 2 µg/mL nicotinamide adenine dinucleotide (NAD) was the liquid broth. Mueller Hinton agar supplemented with 5% defibrinated sheep blood (MHA) was used to grow *P. multocida* and the liquid medium was Cation Adjusted Mueller Hinton Broth (CAMHB). Organisms were incubated in a static incubator at 37$^{o}$C for 18-24 h.

Bacterial counts were determined by serial dilution and spot plate counts. Ten-fold or 100-fold dilutions were carried out in Phosphate Buffered Saline. Three 10 µL drops of the appropriate dilutions were dropped onto the agar surface and allowed to dry for 10 min

before incubating for 24 h. The mean CFU count for each 10 µL was determined, multiplied by 100 and then multiplied by the dilution factor to obtain the initial CFU/mL.

*2.4 Minimum Inhibitory and Minimum Bactericidal Concentrations*

MICs were determined by microdilution for six isolates each of *A. pleuropneumoniae* and *P. multocida*, in accordance with CLSI guidelines, except for: (a) using five sets of overlapping two-fold serial dilutions to increase accuracy; (b) making determinations in serum as well as broth; and (c) growing cultures to 0.5 McFarland Standard (approximately 1-2x$10^8$ CFU/mL) and this was diluted ten-fold to obtain a starting inoculum of 2x$10^7$ CFU/mL. This is higher than, and therefore also a deviation from the CLSI guidelines, which recommend a starting count of 5x$10^5$ CFU/mL. The higher count was selected to provide a medium to heavy microbial load.

Marbofloxacin, media and culture were added successively to each well of 96-well plates. Plates were sealed and incubated statically at 37$^o$C for 24 h. Spot plate counts were prepared immediately after plate inoculation. Tests on each isolate were undertaken in triplicate. Control ATCC isolates were used at a count of 5x$10^5$ CFU/mL as per CLSI guidelines. A positive control well contained medium and pathogen only and a negative control contained medium and marbofloxacin solution. Blank controls contained medium only. For MBC, wells were examined for growth to determine MIC and, in addition to that well, five subsequent concentrations higher than MIC were examined by spot plating. This indicates a 3$\log_{10}$ reduction in inoculum count.

*2.5 Mutant Prevention Concentration*

After growing fresh cultures on agar, approximately 100 single colony forming units (CFU) were used to inoculate culture from plates into a volumetric flask containing 200 mL of pre-warmed broth. This was incubated statically overnight at 37$^o$C. Next day, 1 mL of culture

was added to 9 mL of broth and placed in an orbital incubator for 4 h at 37$^{o}$C and 180 rpm. After 4 h, the bacterial suspension yields 1-2x10$^{11}$ CFU/mL. A spot plate was used to confirm inoculum density.

Final drug concentrations were 1, 2, 4, 8, 16, 32, 64 and 128 multiples of MIC for each of six isolates of each species. These ranges were narrowed down two further times, for example if MPC was 64xMIC the next range would be 32, 36, 40, 44, 48, 52, 56, 60 and 64 x MIC, and the final range if the MPC was 36xMIC would be 32, 32.5, 33, 33.5, 34, 34.5, 35, 35.5 and 36xMIC. Five hundred µL of marbofloxacin solution were applied to cold, dry agar plates and left to dry. Culture (100µL) was added to the plate and allowed to dry. Plates were incubated at 37$^{o}$C for 72 h and checked for growth every 24 h. MPC was the lowest marbofloxacin concentration inhibiting bacterial growth completely after 72 h incubation.

A test was performed to validate the modified MPC method used; the results were identical to those obtained using the method described by Blondeau (2009) (data not shown).

*2.9 Data analysis*

Data were recorded on Microsoft excel, processed using GraphPad Prism v6 and analysed using IBM SPSS Statistics 22 by Kruskal-Wallis H with *post-hoc* Mann-Whitney U test.

## 3. Results

*3.1. Protein binding*

The marbofloxacin concentration curve was linear up to 10 µg/mL. The mean (SD) percentage protein binding was 49.4 (9.61) and was independent of concentration (Table 1). For three batches of serum, SDs were in the range 0.84-1.45 for concentrations of 0.05 to 10 µg/mL and in the range 8.99 to 16.0 for lower concentrations.

*3.2.Minimum Inhibitory, Minimum Bactericidal and Mutant Prevention Concentrations*

Table 2 presents data as geometric means (SD) for MIC, MBC and MPC for broth, serum and serum values corrected for protein binding. Table 3 indicates serum:broth ratios and fraction unbound (fu) serum:broth ratios for MIC, MBC and MPC.

For *P. multocida* MIC, the coefficient of variation ranged from 6-44% for the three repeat estimates in broth and between 0-63% in serum. The greatest variation was seen for the isolates with higher MIC values, and the lowest variations between the three repeats were for isolates with low MIC values. Nevertheless, even when variation percentage appears high, in reality, this roughly equates to a standard doubling dilution. For example, for 63% coefficient of variation in serum, the less susceptible isolate out of six had a lower MIC value of 0.03 µg/mL and higher MIC value of 0.06 µg/mL. Similarly, for *A. pleuropneumoniae*, the coefficient of variation ranged from 0-13% for the three estimates in broth and between 0-22% in serum.

The mean serum:broth MIC ratio for *A. pleuropneumoniae* was 0.79:1 and for *P. multocida* it was 1.12:1. The growth medium thus did not significantly affect MIC for either organism. However, after correcting serum values for protein binding, the fu serum:broth ratio was 0.40:1 for *A. pleuropneumoniae* and 0.50:1 for *P. multocida*; fu serum MICs were significantly lower than broth MICs ($P<0.01$ for *A. pleuropneumoniae* and $P<0.05$ for *P. multocida*) indicating approximately two-fold greater potency of marbofloxacin in serum. For MPC the fu serum:broth ratios were similar to fu serum:broth MIC ratios; 0.50:1 (*A. pleuropneumoniae*, $P<0.05$) and 0.67:1 (*P. multocida*, $P>0.05$).

MBC:MIC ratios were higher for serum than broth for both organisms (Table 4), whereas MPC:MIC ratios were not significantly different for serum compared with broth. For *A. pleuropneumoniae*, the MPC ranged from 11 to 19xMIC in broth and 12-20xMIC in serum.

Similar results were obtained for *P. multocida* in broth (12-20xMIC) and serum (12-20xMIC). For *A. pleuropneumoniae* the MPC:MIC ratio was 13.7:1 in broth and 17.2:1 in serum, and corresponding values for *P. multocida* were 14.2:1 and 18.8:1.

## 4. Discussion

### *4.1 Growth medium and drug potency*

For some drug classes, potency differences between artificial broths and biological fluids may be small and may therefore not significantly impact on dose determination, provided the non-protein bound plasma/serum drug concentration is corrected for (Gonzales et al.,2013; Toutain et al., 2016). For other drug classes, however, differences between broths and biological fluids may be large (Brentnall et al., 2012, 2013; Toutain et al., 2016). Honeyman et al. (2015) compared potencies of several tetracyclines in broth and a 50% broth:50% serum mixture and established marked differences in MIC for the two growth matrices, depending on both chemical structure and bacterial species. Brentnall et al. (2012) reported, after correction for serum protein binding, for a calf isolate of *Mannheimia haemolytica*, a MIC in serum 6 times *greater* than the broth MIC for oxytetracycline. In stark contrast, Toutain et al. (2016) reported, again after correction for serum protein binding, MICs some 80-fold *smaller* in calf serum compared to broth for tulathromycin for *M. haemolytica* and *P. multocida* isolates (six per species) from calves. Furthermore, Zeitlinger et al. (2011) commented "in order to be able to extrapolate data from various models to *in vivo* situations, models should always attempt to mimic physiological conditions as closely as possible". Therefore, the quantitative determination of pharmacodynamic indices, not only with improved accuracy but also in biological matrices, must be regarded, for some drug classes, as appropriate in

applying PK/PD integration and modelling methods to dose determination in subsequent studies.

For human isolates of *Streptococcus pneumoniae*, serum:broth MIC ratios for four fluoroquinolones, ciprofloxacin, levofloxacin, trovafloxacin and moxifloxacin, were 4:1, 1:1, 8:1 and 2:1, respectively (Balcabao et al., 2001), indicating matrix variability between fluoroquinolones for a single bacterial species. Similarly, for calf isolates of *P. multocida*, bovine serum:MHB MIC ratios for marbofloxacin ranged from 1:1 to 7:1 and for *M. haemolytica* the ratio ranged from 0.012:1 to 2.5:1 (Shan et al., 2014; Illambas et al., 2013; Potter et al., 2013). These data from several sources suggest: first, that serum MIC values should be considered on both a drug-by-drug and bacterial species-by-species basis to allow for the inactive protein bound fraction; and second, corrected serum values may not be the same as, and therefore might be used in preference to, the broth MIC for application to prediction of dosage for some drugs. Even better, if available, would be values of MIC determined in biophase fluids, in this instance, pulmonary epithelial lining fluid (PELF).

MIC, MBC and MPC are widely used indicators of antimicrobial drug activity and were used in this study to compare marbofloxacin potency for the pig pneumonia pathogens, *P. multocida* and *A. pleuropneumoniae*, in two matrices, pig serum and CLSI recommended broths. Protein binding is a major factor (and unfortunately often the only factor) considered in seeking to explain medium differences in potency; only free drug is microbiologically active (Wise, 1986; Zeitlinger et al., 2004, 2008). Thus, a higher drug concentration is predicted for the same level of inhibition as in broths, in which albumin concentrations are generally very low. Protein binding of drugs is species dependent; previous investigators have reported protein binding values for marbofloxacin of 15-28% (dogs) and less than 30% (calves) (Aliabadi and Lees, 2002; Bidgood and Papich, 2005).

Protein binding of marbofloxacin in pig serum was 49% in this study. This value is higher than a non-literature estimate of less than 10% for pig serum, though there may be variation between different manufactures/products. (NOAH Compendium, 2016). The value reported in the present study was obtained using a well recognised method, previously used in this laboratory. Moreover, percentage binding was independent of concentration over a wide range (0.0025-10 µg/mL) and was relatively consistent (range of 45.1 to 59.2 over the concentration 0.01 to 10 µg/mL and small/moderate SD) for three separate batches of pig serum. Nevertheless, drug binding to serum protein can vary widely with methodology (Gonzalez et al., 2013) and, for a single drug, intra-species differences have been reported. An example is oxytetracycline in calves – reported literature percentage binding values were 53 (Lees), 50 (Pilloud) and 18.6 (Ziv). Therefore, in future studies, it will be important to determine protein binding of marbofloxacin in pig serum obtained from different sources, differing breeds, ages and indeed between healthy and diseased animals.

For both organisms investigated, correcting the serum MIC for protein binding yielded fu serum MICs significantly less than broth MICs. The fu serum:broth MIC ratio for *A. pleuropneumoniae*, predicted by correction for binding to be 1:1, was 0.40:1. Consequently, correction of serum MIC for protein binding is necessary but not sufficient for determination of potency differences between the two matrices. A similar significant difference, fu serum:broth ratio=0.50:1, was obtained for *P. multocida.* Therefore, for these organisms, dosage prediction from *in vivo* pharmacokinetic data and *in vitro* broth MIC data, would be approximately twice as high as that based on serum MICs. In reporting these potency differences between serum and broth, after correction for binding to protein in serum, it should be noted that serum bound drug will be released over time, extending the duration of action of the drug.

Also of importance for prediction of dosage and determination of efficacy is drug concentration in the biophase. Marbofloxacin distributes extensively to most tissues (including the lung), at higher concentrations than in serum/plasma. As discussed by Gonzalez et al. (2013) and Foster et al. (2016) disease and drug-related factors contribute to differential tissue distribution. For bacterial infections in the pig lung, the biophase is pulmonary epithelial lining fluid (PELF). In this regard, the data of Foster et al. (2016) for another fluoroquinolone, enrofloxacin, in calves may be noted. They reported area under curve values (h*µg/mL) of 17.79 (plasma), 8.89 (interstitial fluid) and 4.03 (PELF).

One factor, potentially accounting for differences in MIC between broths and serum corrected values, is differing bacterial growth rates. Zeitlinger et al. (2008) compared growth curves, in the absence of antimicrobial drugs, of *S.aureus* and *Pseudomonas aeruginosa* in Mueller Hinton Broth (MHB) and serum. Slower logarithmic growth was obtained for both species in serum compared to broth, and this might be expected to provide more rapid kill in serum for a given drug concentration, as a consequence of a smaller microbial challenge. Studies in our laboratory have similarly established slower growth rates in pig serum compared to broth for *P. multocida* and *A. pleuropneumoniae* (Dorey et al., submitted).

Other possible explanations accounting for influence of matrix on MIC were considered by Korz et al. (1995); they suggested that microbial growth might be limited by acidic growth conditions. In the present study, the growth medium became slightly more acidic during the logarithmic growth phase for both broth and serum (data not shown). In further studies, other factors potentially accounting for serum/broth differences described in this paper were investigated (Dorey et al., 2016)

It is important to emphasise that the differences in MIC (and indeed MBC and MPC) between serum and broth reported in this study do not provide a rationale for abandoning broths by

diagnostic laboratories in future studies. This would be impractical and unnecessary. Rather, the present data suggest only that, for marbofloxacin and the two bacterial species studied, it will be possible to apply a scaling factor, to bridge between MICs in broths and pig serum in calculating PK/PD (AUC/MIC) breakpoints, which can be used in predicting dosages, such predictions, in turn, to be confirmed (or not) first in disease model studies and finally in clinical trials.

*4.2 Mutant Prevention Concentration*

Dong et al. (1999) proposed MPC as an *in vitro* indicator of the propensity of an antimicrobial drug to prevent the selection of resistant sub-populations, when high-density bacterial populations were exposed to a range of concentrations. The application of MPC to determine a dosing regimen that avoids emergence of resistance has been proposed (Zinner et al., 2003; Boerlin and Reid-Smith, 2008; Courvalin, 2008). The concept has become widely recognised, because bacterial load during infection may frequently be greater than the inoculum count of $5x10^5$ CFU/ml, defined by CLSI and EUCAST for standardized MIC tests. The initial count in the present MPC studies was $1\text{-}2X10^{11}$ CFU/mL, considerably higher than CLSI recommended count for MIC and even higher than the MIC count used in this investigation of $2x10^7$ CFU/mL, by a factor of $10^4$ CFU/mL.

MPC:MIC ratios in broth and serum were 13.7:1 and 17.2:1, respectively, for *A. pleuropneumoniae*. For *P. multocida*, corresponding ratios were 14.2:1 and 18.8:1, broth serum differences which were not significantly different. These MPC:MIC ratios were less than in some published studies but greater than in others. For example, for *M. haemolytica* a 62.5:1 ratio was reported for enrofloxacin, based on $MIC_{90}$ (Blondeau et al., 2012). For *Mycobacterium smegmeatis* the MPC:$MIC_{99}$ ratio was 22:1 for moxifloxacin, whereas for the same drug a 3.2:1 ratio was reported for *S. aureus* (Blondeau et al., 2012). From these and the

present findings, it seems clear that, as for MICs, MPCs must be determined on a drug-by-drug, matrix-by-matrix and bacterial species-by-species basis.

Application of MPC, linked to pharmacokinetic profiles, provides a strategy for designing dosages to minimise emergence of resistance. Schneider et al. (2014) reported plasma marbofloxacin concentrations in the pig exceeding 0.6 and 1.0 µg/mL for 24 h after intramuscular doses of 4 and 8 mg/kg, respectively, while concentrations exceeded 1.0 and 3.0 µg/mL for 12 h for these two doses. For the 8 mg/kg dose, plasma maximum concentration was 6.30 µg/mL. As mean serum MPCs in this study were 12.6 and 0.75µg/mL for *A. pleuropneumoniae* and *P. multocida*, respectively, it is more likely that a dose to avoid emergence of resistance is attainable for the latter species.

In contrast with the similarity of MPC:MIC ratios for broth and serum for both bacterial species, MBC:MIC ratios were somewhat higher in serum than in broth, 3.80:1 versus 2.47:1 for *A. pleuropneumoniae* and 4.89:1 versus 1.71:1 for *P. multocida.* The possible causes of these trends may be that at low antimicrobial drug concentrations where the isolates are susceptible, these tests may be influenced by the culture media and environmental conditions, however, these external conditions may not be so influential at high drug concentrations.

*4.3 Conclusions*

The present study reports comparative MIC, MBC and MPC data for two growth matrices, broth and pig serum for the pig pneumonia pathogens, *A. pleuropneumoniae* and *P. multocida*. Significant differences were obtained after correction of serum data for marbofloxacin binding to serum protein. In future studies, the present data can be used in dose prediction studies, using a scaling factor to bridge between serum and broth differences.

**Summary**

Moderate growth matrix dependent differences in MIC were demonstrated for marbofloxacin for two pathogenic bacterial species harvested from pigs, *A. pleuropneumoniae* and *P. multocida*. Correction of MIC serum data for drug protein binding revealed significant differences from broth MICs, whereby the active (unbound serum) concentration was 2-fold lower than predicted. The data indicate that correction for drug binding to serum protein is necessary but not sufficient to explain matrix differences in marbofloxacin potency. For both growth media, the use of five overlapping sets of two-fold dilutions increased the accuracy of MIC, MBC and MPC determinations. For *A. pleuropneumoniae* and *P. multocida*, serum MPCs were 17-19 fold higher than MICs.

**Acknowledgements**

The study was funded by BBSRC and Norbrook Laboratories Ltd. The lead author was a BBSRC CASE Scholar. A. Pridmore, Don Whitley Scientific and A. Rycroft, Royal Veterinary College, supplied bacterial isolates. Dr. Z. Cheng provided support for protein binding studies.

## Conflict of interest

None of the authors of this paper have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper. Norbrook Laboratories Ltd. co-sponsored this work with BBSRC. During the last 5 years the authors interests have included the following: P. Lees (consultancy advice supplied to Bayer

Animal Health, Norbrook Laboratories Ltd. and Pfizer Animal Health); and S. Hobson (employee of Norbrook Laboratories Ltd.).

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**Table 1**

Marbofloxacin serum protein binding

| Concentration (µg/mL) | Mean percentage binding (SD) |
|---|---|
| 10 | 55.7 (0.84) |
| 5 | 49.4 (1.08) |
| 1 | 59.2 (0.84) |
| 0.5 | 50.8 (1.39) |
| 0.1 | 51.3 (1.45) |
| 0.05 | 45.1 (1.43) |
| 0.01 | 35.0 (15.98) |
| 0.005 | 41.2 (8.99) |
| 0.0025 | 56.7(11.08) |
| Mean | 49.4(9.61) |

Serum protein binding over concentration range 0.0025 to 10µg/mL. Values are mean (SD) for three batches of serum for each concentration.

**Table 2**

*A. pleuropneumoniae* (APP) and *P. multocida* (PM) MIC, MBC and MPC for broth, serum and fu serum†.

| Organism | Medium | MIC | MBC | MPC |
|---|---|---|---|---|
| APP | Broth | 0.90 (0.24) | 2.21 (0.43) | 12.68 (2.72) |
| | Serum | 0.71 (0.22) | 2.68 (0.49) * | 12.60 (4.73) |
| | fu Serum | 0.36 (0.11)** | 1.36 (0.25)* | 6.38(3.72)* |
| PM | Broth | 0.04 (0.06) | 0.06 (0.10) | 0.57 (0.74) |
| | Serum | 0.04 (0.40) | 0.21 (0.76) * | 0.75 (8.00)* |
| | fu Serum | 0.02 (0.20)* | 0.10 (0.39) | 0.38(1.46) |

†fu serum=serum concentrations corrected for protein binding. Geometric means (SD) of marbofloxacin concentrations for each of six isolates of each organism determined in triplicate. Significant differences of serum and fu serum from broth values: *P<0.05, **P<0.01.

**Table 3**

*A. pleuropneumoniae* (APP) and *P. multocida* (PM) serum:broth and fu serum:broth MIC, MBC and MPC ratios

| Organism | Ratio | MIC | MBC | MPC |
|---|---|---|---|---|
| APP | Serum:broth | 0.79:1 | 1.21:1 | 0.99:1 |
| | fu Serum:broth | 0.40:1 | 0.62:1 | 0.50:1 |
| PM | Serum:broth | 1.12:1 | 3.20:1 | 1.32:1 |
| | fu Serum:broth | 0.50:1 | 1.67:1 | 0.67:1 |

†fu serum=serum concentrations corrected for protein binding

**Table 4**

*A. pleuropneumoniae* (APP) and *P. multocida* (PM) MBC:MIC and MPC:MIC ratios for artificial broth and pig serum

| Organism | Medium | MBC:MIC | MPC:MIC |
|---|---|---|---|
| APP | Broth | 2.47 : 1 | 13.7 : 1 |
| | Serum | 3.80 : 1 | 17.2 : 1 |
| PM | Broth | 1.71:1 | 14.2:1 |
| | Serum | 4.89:1 | 18.8:1 |

## Part 1: Highlights

- Pharmacodynamic indices of potency of marbofloxacin (MIC, MBC, MPC) were bacterial species and growth matrix dependent
- Serum/broth differences in potency may influence PK/PD approaches to dose determination in future studies