Research in Veterinary Science

Factors influencing the potency of marbofloxacin for pig pneumonia pathogens *Actinobacillus pleuropneumoniae* and *Pasteurella multocida*

L. Dorey*[1]

S. Hobson[2]

P. Lees[1]

[1]The Royal Veterinary College, Department of Comparative Biological Sciences, Hawkshead Campus, Hatfield, Herts, AL9 7TA, United Kingdom

[2] Norbrook Laboratories Ltd., Newry, Co. Down, BT35 6QQ, Northern Ireland

*Corresponding author. Tel: +44 01707666477. fax: +44 1707 666659 *E.mail address*: ldorey@rvc.ac.uk. (L Dorey)

Short title: Marbofloxacin and pig pathogens

Abstract

For the pig respiratory tract pathogens, *Actinobacillus pleuropneumoniae and Pasteurella* multocida, Minimum Inhibitory Concentration (MIC) of marbofloxacin was determined in recommended broths and pig serum at three inoculum strengths. MICs in both growth matrices increased progressively from low, through medium to high starting inoculum counts, 10^4, 10^6 and 10^8 CFU/mL, respectively. *P. multocida* MIC ratios for high:low inocula were 14:4:1 for broth and 28.2:1 for serum. Corresponding MIC ratios for *A. pleuropneumoniae* were lower, 4.1:1 (broth) and 9.2:1 (serum). MIC high:low ratios were therefore both growth matrix and bacterial species dependent. The effect of alterations to the chemical composition of broths and serum on MIC were also investigated. Neither adjusting broth or serum pH in six increments over the range 7.0 to 8.0 nor increasing calcium and magnesium concentrations of broth in seven incremental steps significantly affected MICs for either organism. In time-kill studies, the killing action of marbofloxacin had the characteristics of concentration dependency against both organisms in both growth matrices. It is concluded that MIC and time-kill data for marbofloxacin, generated in serum, might be preferable to broth data, for predicting dosages of marbofloxacin for clinical use.

Keywords:

Marbofloxacin; Minimum Inhibitory Concentration; Time-kill; *Pasteurella multocida; Actinobacillus pleuropneumoniae*

1. Introduction

The most widely used parameter of antimicrobial drug potency is Minimum Inhibitory Concentration (MIC). This is the lowest (fixed) drug concentration which inhibits bacterial growth in vitro under standard conditions, after incubation for a fixed time. Guidelines and standards have been set by the European Union Committee on Antimicrobial Sensitivity testing (EUCAST) and the Clinical Laboratory Standards Institute (CLSI). Their methods require use of a specific broth for each organism (formulated to facilitate /optimise bacterial growth) and are conducted using two-fold dilutions. Each test incorporates control organisms of known MIC. The adoption of CLSI and EUCAST guidelines and standards ensures that data generated is consistent between individual experimenters, between laboratories and across countries (Papich 2013). From a clinical use perspective, the principal objective in generating MIC data for each drug against each pathogenic organism harvested from each animal species is its application to determine dosage regimens that optimise bacterial kill and minimise opportunities for the emergence of resistance. For this purpose the following considerations are paramount:

- EUCAST and CLSI standardised MIC data are generated in broths, such as Cation Adjusted Mueller Hinton Broth (CAMHB), each formulated to facilitate growth *in vitro*, the composition differing (for some constituents markedly) from biological fluids in general and from the biophase in particular, in respect of chemical, immunological and cellular constituents, so that they might not be representative of bacterial growth conditions *in vivo*;

- EUCAST and CLSI standardised MICs are determined using two-fold dilutions and are subject to good precision but potentially high inaccuracy - for an isolate MIC of say 4 µg/mL, the next lower MIC is 2 µg/mL and the "true" MIC might be 2.01

µg/mL; from dose determination perspectives it is accuracy rather than which is required;

- MICs provide a measure of potency for a fixed concentration over a fixed time period, whereas in clinical use drug concretions decrease steadily after intravenous administration or increase to a maximum and then decrease, as absorption is balanced by elimination after non-vascular dosing;

- MIC gives no indication of the time course of killing action , which may be concentration-, time- or co-dependent and this killing pattern may differ for a single drug against differing microbial species;

- MIC is determined using a fixed inoculum count of $5x10^5$ CFU/mL under EUCAST and CLSI guidelines, whilst bacterial load, on which rate and extent of bacterial kill is likely to depend, may be much higher or lower and vary with time *in vivo*;

- *in vivo*, in diseased but immunocompetent animals, bacteriological cure will be assisted by natural body defences.

To partially address the above considerations, in relation to correlating pharmacokinetic and pharmacodynamic data to predict dosages, we have used five sets of overlapping two-fold dilutions in previous studies; this reduces the inaccuracy from approaching 100% to not exceeding 20% (Aliabadi and Lees, 2001; Sidhu et al., 2010). Also, to enable comparisons between broth and biological fluids, as growth matrices for bacteria, and to evaluate possible differences between them, previous studies have been undertaken in serum, plasma and inflammatory exudate (Aliabadi and Lees, 2001, 2002; Nightingale and Murakawa 2002; Zeitlinger et al., 2004, 2008, 2011; Sidhu et al., 2010). A future development might be

generation of MIC data in biophase fluids, which might be serum, urine (of varying pH), milk, prostate and pulmonary epithelial lining fluid etc.,

For some drug classes, potency differences between broths and biological fluids are small and have no significant impact on dose prediction; provided the inactive protein bound plasma/serum drug concentration is known and corrected for. For other drug classes, however, differences between broths and biological fluids may be large. In a recent study, several tetracyclines were compared for MICs in broth and 50% broth:50% mouse serum as matrices (Honeyman et al., 2015). For a strain of *Strep. pneumoniae,* MICs were identical for five compounds but, with added serum, 2-4 fold increases were obtained for five further compounds, and MIC was increased 32-fold for one compound. In contrast, for a strain of *Staph. aureus,* MIC was increased in the broth:serum combined matrix relative to broth for all 12 compounds investigated and for seven the increase was in the range 8-128-fold. Likewise, Brentnall et al. (2012) reported for a calf isolate of *Mannheimia haemolytica* a MIC in serum 19 times *greater* than the broth MIC for oxytetracycline. In contrast, Illambas et al. (2009) and Toutain et al. (2016) reported MICs some 50-fold *smaller* in calf serum compared to broth for tulathromycin for *Mannheimia haemolytica* and *Pasteurella multocida* isolates from calves, and this difference was some 80-fold when serum values were corrected for protein binding. Zeitlinger et al. (2011) have proposed "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". Hence, the quantitative determination of pharmacodynamic indices with improved accuracy and in biological matrices should be regarded, for some drug classes, as appropriate in applying PK/PD integration and modelling methods to dose determination.

To optimise clinical efficacy and minimise the emergence of resistance to antimicrobial drugs, a further consideration is dosage required for differing pathogen loads. For

metaphylaxis and treatment early in the course of disease, when the pathogen load is low or absent, many drugs, at recommended dose rates, will prevent or cure disease, in support of natural body defences. The major challenge for antimicrobial drugs, however, is to provide a bacteriological cure and avoid emergence of resistance, when pathogen numbers in the biophase are high. For this reason, a high starting inoculum count of approximately 10^7cfu/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 aims of this investigation were: (1) for marbofloxacin and six isolates each of *Actinobacillus pleuropneumoniae* and *P. multocida* (respiratory pathogens in the pig) to compare MICs in two matrices, CLSI recommended broths and pig serum, with levels of inoculum count, low, intermediate and high; (2) for two isolates of each species to investigate the effect on MIC of (a) varying the initial inoculum pH of serum and broth over the range 7.0 to 8.0, (b) increasing the calcium and magnesium concentrations of broth and (c) addition of serum to broth in varying proportions; (3) to establish time-kill curves in serum and broth for six isolates *A. pleuropneumoniae* and *P. multocida* of eight multiples of MIC.

2. Materials and Methods

2.1 Bacterial isolates

Twenty isolates of *P. multocida* were supplied by Don Whitley Scientific (Shipley, West Yorkshire, BD173SE, UK). This company also supplied three ATCC reference strains for use in all MIC tests; *A. pleuropneumoniae* ATCC 27090, *Enterococcus faecalis* ATCC 29212and *E.coli* ATCC 25922. Eight isolates of *A. pleuropneumoniae* were supplied by A. Rycroft (Royal Veterinary College, Hatfield, Herts., UK). All 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 5sec, left to cool for 12h and then boiled again for a further 5sec.

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 two-fold dilutions; and (3) then, selection of isolates with the highest and lowest broth MICs plus four randomly selected isolates with intermediate MICs. This pre-selection procedure ensured that all isolates could be used in further MIC and time-ill investigations in broth and serum.

2.2 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 2µg/mL nicotinamide adenine dinucleotide (NAD) was the liquid broth. This was selected in preference to veterinary fastidious medium (VFM) as it was consistently better at supporting bacterial growth and without the formation of blood pellets in VFM determination of MIC endpoint was easier for user. Mueller Hinton agar supplemented with 5% defibrinated sheep blood (MHA) was used to grow *P. multocida* and the liquid medium was 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 10µL drops of the appropriate dilutions were dropped onto the agar surface and allowed to dry for 10min before incubating. After 24 h incubation, the mean CFU count for each 10µL was determined, multiplied by 100 and then multiplied by the dilution factor to obtain the original CFU/mL.

2.3 Minimum Inhibitory Concentrations

MICs were determined by microdilution for six isolates each of *A. pleuropneumoniae* and *P. multocida*, in accordance with CLSI guidelines, except for (1) using five sets of overlapping two-fold serial dilutions to reduce inaccuracy of individual isolate estimates from up to 100% to not greater than 20%; (2) comparing serum with broth; (3) using Columbia broth supplemented with 2µg/mL nicotinamide adenine dinucleotide (NAD) as the liquid broth; and (4) use of starting inocula of three strengths. These were high ($1.5.10^8$CFU/mL), medium ($1.5.10^6$CFU/mL) and low ($1.5.10^4$CFU/mL). A 0.5 McFarland standard was made to provide a count of $1.5x10^8$CFU/mL (high). Two 1:10 dilutions provided a count of $1.5x10^6$CFU/mL (medium). Two further 1:10 dilutions gave counts of $1.5x10^4$CFU/mL (low). MICs were determined in broth and serum, for six isolates of each species, triplicate estimates per isolates.

Marbofloxacin, media and culture were added successively to each well of 96-well plates and plates sealed and incubated statically at 37^{o}C for 24h. 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 $5x10^5$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.

2.4 Effect of varying pH and divalent cation concentrations on MIC

Studies were conducted on two isolates each of *P. multocida* and *A. pleuropneumoniae* to investigate the influence on MIC of: (1) varying inoculum pH (broth and serum); (2) increased concentrations of Ca^{++} and Mg^{++} (broth). To determine the effect of pH on MIC, the starting pH was adjusted to 7.0, 7.2, 7.4, 7.6, 7.8 or 8.0, using hydrochloric acid (1M) or sodium hydroxide (1M).

Calcium and magnesium ion concentrations of CAMHB, to meet CLSI standards, are in the ranges 20 to 25 mg/L Ca^{++} and 10 to 12.5 mg/L Mg^{++}. The concentrations of these cations were increased by addition of $CaCl_2$ and $MgCl_2$ solutions with seven adjustments (increased cation concentrations (mg/L) : 1 (0mg Ca and 0mg Mg); 2 (5mg Ca and 3mg Mg); 3 (10mg Ca and 6mg Mg); 4 (15mg Ca and 10mg Mg); 5 (20mg Ca and 12mg Mg) ; 6 (25mg Ca and 15mg; and 7 (30mg Ca and 18mg Mg). This test was repeated for *P. multocida* and *A. pleuropneumoniae*, although it is noted that CAMHB is not the recommended broth for *A. pleuropneumoniae*.

2.5 *Time-kill curves*

Time-kill curves were generated for six isolates each of *A. pleuropneumoniae* and *P. multocida* in both broth and serum. Two to 3 colonies of each isolate were added to 5 mL broth or serum and incubated overnight in an orbital incubator. Fifty µL of culture were diluted 1:50 in pre-warmed broth or serum and incubated statically for 1 h at 37°C. This was compared to a 0.5 McFarland Standard and diluted ten-fold to obtain the starting inoculum, consistent with MIC determinations, at $2x10^7$CFU/mL, and confirmed by colony counts. Concentrations comprising eight multiples of MIC (0.25, 0.5, 1, 1.5, 2, 4, 6 and 8x) were prepared in pre-warmed broth or serum. Ten µL of culture were used to inoculate the dilutions to give a 1 mL final volume.

Fifty µL of each culture were sampled and viability determined through serial dilution and spot plates at nine time points; 0, 0.25, 0.5, 0.75, 1, 2, 4, 8 and 24h. No organism was added as a negative control, whereas in positive controls no drug was added. Spot plates were examined for contamination and biochemical identification methods were applied for suspect colonies. Measurements were conducted in triplicate for six isolates of both organisms in broth and serum. The lower limit of quantification (LLOQ) was 33CFU/mL.

2.6 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 Influence of inoculum count on MIC

MICs were inoculum size dependent for both organisms, increasing progressively with inoculum count, in both media (Tables 1 and 2). For *P. multocida*, MIC ratios high:low inocula were significant ($P<0.001$) for broth (14.4:1) and serum (28.2:1). Ratios high:medium inocula were also significant for both media, 7.21:1 (broth) and 11.2:1 (serum) ($P<0.01$). For *A. pleuropneumoniae* high:low inocula ratios were somewhat lower but also statistically significant ($P<0.01$) for broth (4.1:1) and serum (9.2:1). Ratios high:medium inocula were also significant for both media.

The data revealed a second important effect of initial inoculum load on MICs. The serum:broth MIC ratio was higher for the high inoculum count (1.23:1) than for the low starting count for which the ratio was 0.63:1.

3.2 Influence of pH and cation concentrations on MIC

Alteration of pH over the range 7.0 to 8.0, in six increments, did not affect MICs for either *P. multocida* (Fig.1) or *A. pleuropneumoniae* (Fig.2) in either broth or serum. In broth, increasing concentrations of Ca^{++} and Mg^{++} likewise did not significantly affect MICs for either organism (Fig.3).

3.4 Time-kill curves

Time-kill curves for eight multiples of MIC for six isolates of each species are illustrated in Figs. 1 (*P. multocida*) and 2 (*A. pleuropneumoniae*). For *P.multocida*, there was progressive growth inhibition at 1.5 and 2xMIC in both media; 2xMIC produced a $3\log_{10}$decrease in CFU/mL by 2-4 h. In broth, concentrations at and above 4xMIC resulted in virtual eradication at 8h, and at 8xMIC by 24 h in serum. For *A. pleuropneumoniae*, the patterns of growth inhibition were very similar for both media; little inhibition occurred at concentrations less that 1xMIC but 4xMIC produced a $3\text{-}4\log_{10}$ decrease in CFU/mL by 8 h in both media. Virtual eradication (< 33 CFU/mL) occurred in both media at 6xMIC by 8 h and at 8xMIC by 4 h. For both organisms, the action of marbofloxacin in both media was classed as concentration-dependent.

4. Discussion

A previous study from this laboratory reported no significant differences in MIC between broths and pig serum for the pig pneumonia pathogens, *P. multocida* and *A. pleuropneumoniae* (Dorey et al., submitted). However, when serum MICs were corrected for drug binding to serum protein, to allow for the fact that protein bound drugs are microbiologically inactive (Wise, 1986, Zeitlinger, 2011, Gonzalez, 2013) the unbound fraction MIC was approximately 0.5X the broth MIC. This finding indicated that potency was approximately twice as high (MIC lower) in serum compared with broth and therefore that correction for protein binding is necessary but not sufficient. The data suggested that biological fluids such as serum might be more suitable for PK/PD integration and modelling procedures for predicting optimal doses, for example through establishing PK/PD breakpoints.

In addition to protein binding, Zeitlinger et al. (2008) described another factor potentially accounting for differences in MIC between broths and serum, namely differing bacterial growth rates. They compared growth curves, in the absence of antimicrobial drugs, for *Staphylococcus aureus* and *Pseudomonas aeruginosa* in MHB, serum and varying proportions of serum admixed with MHB. For both species, logarithmic growth was slower in serum than broth. This might provide more rapid kill in serum for a given drug concentration, as a consequence of a lesser microbial challenge. Slower growth rates in pig serum compared to broth for *P. multocida* and *A. pleuropneumoniae* were obtained for both species in pig serum compared to broth in our laboratory (Dorey et al., unpublished data).

The above findings stimulated further research, now published in this article. It was clearly established that a high inoculum count (10^8CFU/mL) increased broth MIC by some 14-fold for *P. multocida* compared to a low count of 10^4CFU/mL. Whilst differences might well be predictable, it is important to establish the magnitude of differences, if (as we intend) the data is to be used in PK/PD breakpoint and dose prediction studies for differing pathological loads. Moreover, the difference was growth matrix dependent; the corresponding high:low ratio for serum MIC was 28-fold greater. This matrix dependency lends further weight to the rationale for conducting MIC studies in biological fluids rather than broths for the purpose of determining PK/PD breakpoints. Qualitatively, similar findings were obtained for *A. pleuropneumoniae* but quantitatively they were smaller; thus, high:low MIC ratios were 4.1:1 (broth) and 9.2:1 (serum).

As a further aspect of the data, illustrating the complexity of the interactions between load and matrix, one might note the serum/broth MIC ratios. These differed, depending on inoculum load. For both pathogens, the serum:broth MIC ratio was 1.23:1 for the high inoculum load and corresponding values for the low load were 0.63:1 (*P. multocida*) and o 0.55:1 (*A. pleuropneumoniae*).

In light of the above findings, the present study investigated two of many possible factors affecting MICs, in addition to inoculum load and logarithmic growth rate. For some drug classes (e.g. macrolides/triamilides) there is high pH dependency (Toutain et al., 2016) and for tetracyclines the potential for covalent binding to calcium and/or magnesium ions has been reported (Carlotti et al. (2012). Korz et al. (1995) 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 this study, however, altering pH in increments of 0.2 units between pHs 7.0 and 8.0 did not affect MIC for either organism and both growth matrices. Likewise, increasing in combination broth concentrations of Ca^{++} and Mg^{++} incrementally did not alter MICs.

Finally, our data illustrate the importance of establishing not only the potency of drugs in MIC studies which use, under EUCAST and CLSI guidelines, exposure to constant concentrations for fixed time periods and a single inoculum count in non-biological fluids, but also the potency in biological fluids and the time course of killing action. For marbofloxaxin, against the two pathogens studied, this was shown to be concentration-dependent in serum as well as broth. Time courses of kill were similar but not identical and by 24 h, in both matrices at 2xMIC, 3.5-4$\log_{10}$ reductions in bacterial count had occurred. In future studies it would be relevant to extend these findings by evaluating level of kill at 48h and 72h and also rates of kill at even higher multiples of MIC (Mitchell et al. 2012). These investigations should now be extended to establish drug efficacy and potency with varying concentrations over time using, for example, hollow fibre infection models.

In summary, the present data suggest that MIC values should be considered on drug-by-drug, matrix-by matrix, species-by-species and load-by-load bases, not only to allow for the inactive protein bound fraction but also to allow for the many in vitro differences that can be

applied, in combination with drug pharmacokinetics to prediction of dosage for use in disease models and then clinical trials.

4. Summary

Small but significant growth matrix dependent effects on MIC, MBC and MPCs were demonstrated for marbofloxacin for two pathogenic bacterial species harvested from pigs, *A.pleuropneumoniae* and *P.multocida*. For these indices of potency, correction of MIC serum data for drug protein binding revealed significant differences, whereby the active concentration (unbound serum concentration) was 2-fold lower than predicted, indicating that correction for drug binding to serum protein is necessary but not sufficient. For both growth media, the use of five overlapping sets of two-fold dilutions increased the accuracy of determinations. For *A.pleuropneumoniae* and *P.multocida*, serum MPCs were 17-19 folds higher than MICs. Marbofloxacin was concentration-dependent in its killing action against both pathogens.

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 organization that could inappropriately influence or bias the content of the paper. 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.).

References

Aliabadi, F.S., Lees, P., 2001. Pharmacokinetics and pharmacodynamics of danofloxacin in serum and tissue fluids of goats following intravenous and intramuscular administration. American Journal of Veterinary Research 62, 1979-1989.

Aliabadi, F.S., Lees, P., 2002. Pharmacokinetics and pharmacokinetic/pharmacodynamic integration of marbofloxacin in calf serum, exudate and transudate. Journal of Veterinary Pharmacology and Therapeutics 25, 161-174.

Brentnall, C., Z. Cheng, Q. A. McKellar, and P. Lees, 2012, Pharmacodynamics of oxytetracycline administered alone and in combination with carprofen in calves: Veterinary Record, 171, 273-277.

Carlotti, B.,Cesaretti, A., Elisei, F., 2012. Complexes of tetracyclines with divalent metal cations investigated by stationary and femtosecond-pulsed techniques. Physical Chemistry Chemical Physics, 14, 823-824.

CLSI, 2008. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals: Approved Standard - Third Edition. CLSI document M31-A3.

Dorey, L., Hobson, S., Lees, P., 2016. Potency of marbofloxacin for pig pneumonia pathogens Actinobacillus pleuropneumoniae and Pasteurella multocida: comparison of growth media. Research in Veterinary Science, submitted.

Honeyman, L., Ismail, M., Nelson, M.L., Bhatia, B., Bowser, T.E., Chen, J., Mechiche, R., Ohemeng, K., Verma, A.K., Cannon, E.P., Macone, A., Tanaka, S.K. & Levy, S. 2015. Structure-activity relationship of the aminomethylcyclines and the discovery of omadacycline. Antimicrobial Agents and Chemotherapy, 59, 7044-7053.

Gonzalez, D., Schmidt, S., Derendorf, H., 2013. Importance of Relating Efficacy Measures to Unbound Drug Concentrations for Anti-Infective Agents. Clinical Microbiology Reviews 26(2), 274-288

Illambas, J., Potter, T., Rycroft, A. & Lees, P. (2009) Pharmacodynamics of tulathromycin in vitro and ex vivo against calf pathogens. Journal of Veterinary Pharmacology and Therapeutics 32, 62-66.

Korz, D.J., Rinas, U., Hellmuth, K., Sanders, E.A., Deckwer, W.D., 1995. Simple fed-batch technique for high cell density cultivation of Escherichia coli. Journal of Biotechnology 39, 59-65.

Mitchell, J.D., McKellar, Q.A., McKeever, D.J., 2012. Pharmacodynamics of antimicrobials against Mycoplasma mycoides mycoides small colony, the causative agent of contagious bovine pleuropneumonia. PLoS One 7, e44158.

Nightingale, C.H., Murakawa, T., 2002. Microbiology and pharmacokinetics. Antimicrobial Pharmacodynamics in Theory and Clinical Practice.

Papich, M.G., 2013. Antimicrobials, susceptibility testing, and minimum inhibitory concentrations (MIC) in veterinary infection treatment. Vet Clin North Am Small Anim Pract 43, 1079-1089.

Sidhu, P.K., Landoni, M.F., Aliabadi, F.S., Lees, P., 2010. Pharmacokinetic and pharmacodynamic modelling of marbofloxacin administered alone and in combination with tolfenamic acid in goats. The Veterinary Journal 184, 219-229.

Toutain, P.-L., Potter, T., Pelligand, L., Lacroix, M., Illambas, J., Lees, P. 2016. Standard PK/PD concepts can be applied to determine a dosage regimen for a macrolide: the case of

tulathromycin in the calf. Journal of Veterinary Pharmacology and Therapeutics. doi: 10.1111/jvp.12333.

Wise, R., 1986. The clinical relevance of protein binding and tissue concentrations in antimicrobial therapy. Clinical Pharmacokinetics 11, 470-482.

Zeitlinger, M., Sauermann, R., Fille, M., Hausdorfer, J., Leitner, I., Müller, M., 2008. Plasma protein binding of fluoroquinolones affects antimicrobial activity. The Journal of antimicrobial chemotherapy 61, 561-567.

Zeitlinger, M.A., Sauermann, R., Traunmüller, F., Georgopoulos, A., Müller, M., Joukhadar, C., 2004. Impact of plasma protein binding on antimicrobial activity using time-killing curves. The Journal of antimicrobial chemotherapy 54, 876-880.

Zeitlinger, M. A., Derendorf, H., Mouton, J. W., Cars, O., Craig, W. A., Andes, D., & Theuretzbacher, U., 2011. Protein Binding: Do We Ever Learn? Antimicrobial Agents and Chemotherapy, 55, 3067–3074.

Table 1

P. multocida broth and serum MICs for three inoculum strengths†, inoculum size ratios and serum:broth MIC ratios

Inoculum size	Broth MIC	Serum MIC	Serum:broth ratio
High	0.23 (0.06)	0.28 (0.07)	1.23 : 1
Medium	0.03 (0.02)	0.03 (0.01)	0.79 : 1
Low	0.02 (0.01)	0.01 (0.00)	0.63 : 1
High:low ratio	14.4 :1 **	28.2 : 1 **	
High:medium ratio	7.2 : 1 *	11.2 : 1 *	
Medium:low ratio	2.0 : 1	2.5 : 1	

†: High (1.5 x 10^8CFU/mL), medium (1.5 x 10^6CFU/mL) and low (1.5 x 10^4CFU/mL). MICs are geometric means (SD) of six isolates: triplicate analyses for each. Significant differences between inoculum strengths *P<0.01 **P<0.001.

Table 2

A. pleuropneumoniae broth and serum MICs for three inoculum strengths†, inoculum size ratios and serum:broth MIC ratios

Inoculum size	Broth MIC	Serum MIC	Serum:Broth ratio
High	1.91 (0.18)	2.34 (0.22)	1.23 : 1
Medium	0.77 (0.11)	0.66 (0.05)	0.86 : 1
Low	0.46 (0.04)	0.26 (0.02)	0.55 : 1
High:low ratio	4.1 : 1**	9.2 : 1 **	
High:medium ratio	2.5 : 1*	3.5 : 1*	
Medium:low ratio	1.7 : 1	2.6 : 1	

†: High (1.5 x 10^8CFU/mL), medium (1.5 x 10^6CFU/mL) and low (1.5 x 10^4CFU/mL). MICs are geometric means (SD) of six isolates: triplicate analyses for each. Significant differences between inoculum strengths: *P<0.05 **P<0.01.

Figure 1. MICs for two isolates of *P. multocida* (PM) in broth and serum adjusted to six pH values: means of two isolates and standard deviation for triplicate analyses.

Figure 2. MICs for two isolates of *A. pleuropneumoniae* (APP) in broth and serum adjusted to six pH values: means of two isolates and standard deviation for triplicate analyses.

Figure 3. Marbofloxacin MICs (µg/mL):mean and standard deviation (triplicate analyses)for two isolates each of *P.multocida* and *A.pleuropneumoniae* in CAMHB with increased cation concentrations (mg/L) : 1 (0mg Ca and 0mg Mg); 2 (5mg Ca and 3mg Mg); 3 (10mg Ca and 6mg Mg$^{)}$; 4 (15mg Ca and 10mg Mg$^{)}$; 5 (20mg Ca and 12mg Mg) ; 6 (25mg Ca and 15mg; and 7 (30mg Ca and 18mg Mg$^{)}$.

Figure 4. *P. multocida* growth inhibition over 24 h exposure to 8 multiples of MIC in (A) CAMHB and (B) serum (n=6). Bars=standard deviation. LLOQ= 33CFU/mL.

Figure 5. *A. pleuropneumoniae* growth inhibition over 24 h exposure to 8 multiples of MIC in (A) Columbia broth supplemented with NAD and (B) pig serum (n=6). Bars=standard deviation. LLOQ= 33CFU/mL.

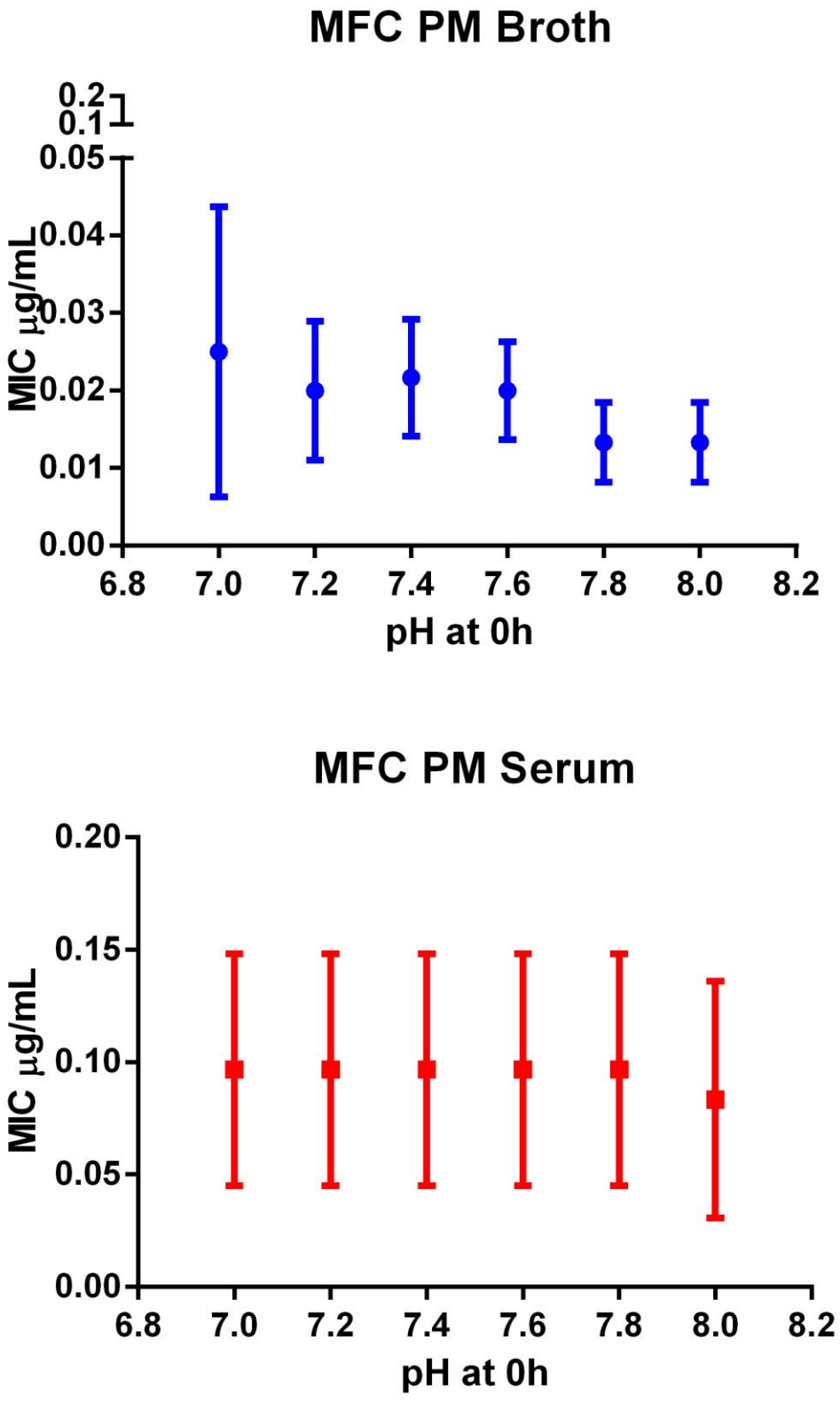

Figure 1

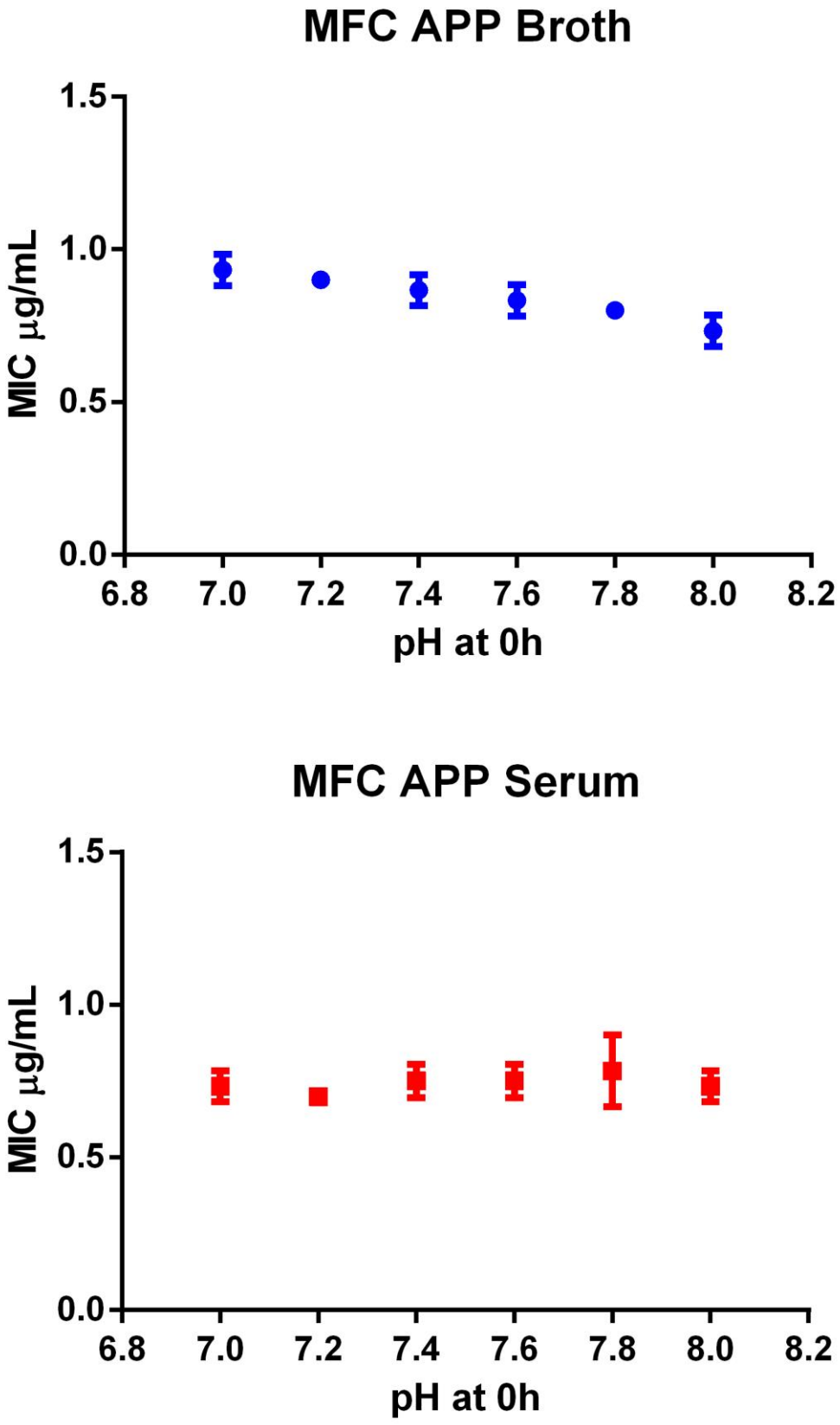

Figure 2

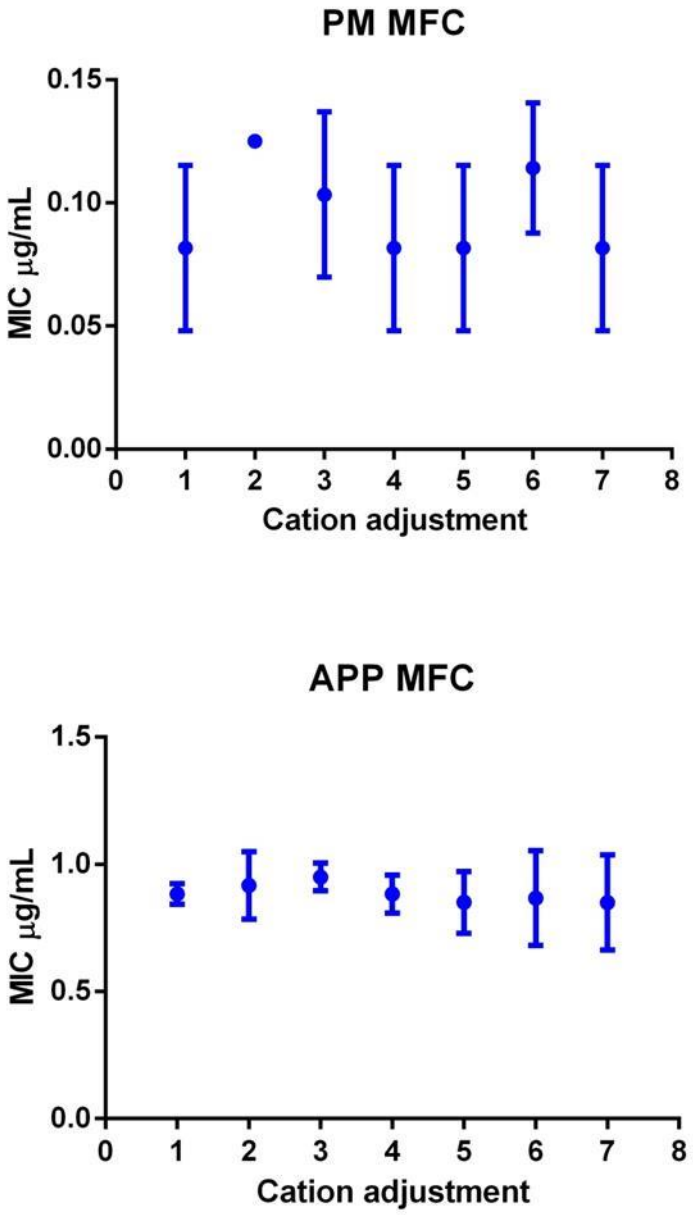

Figure 3

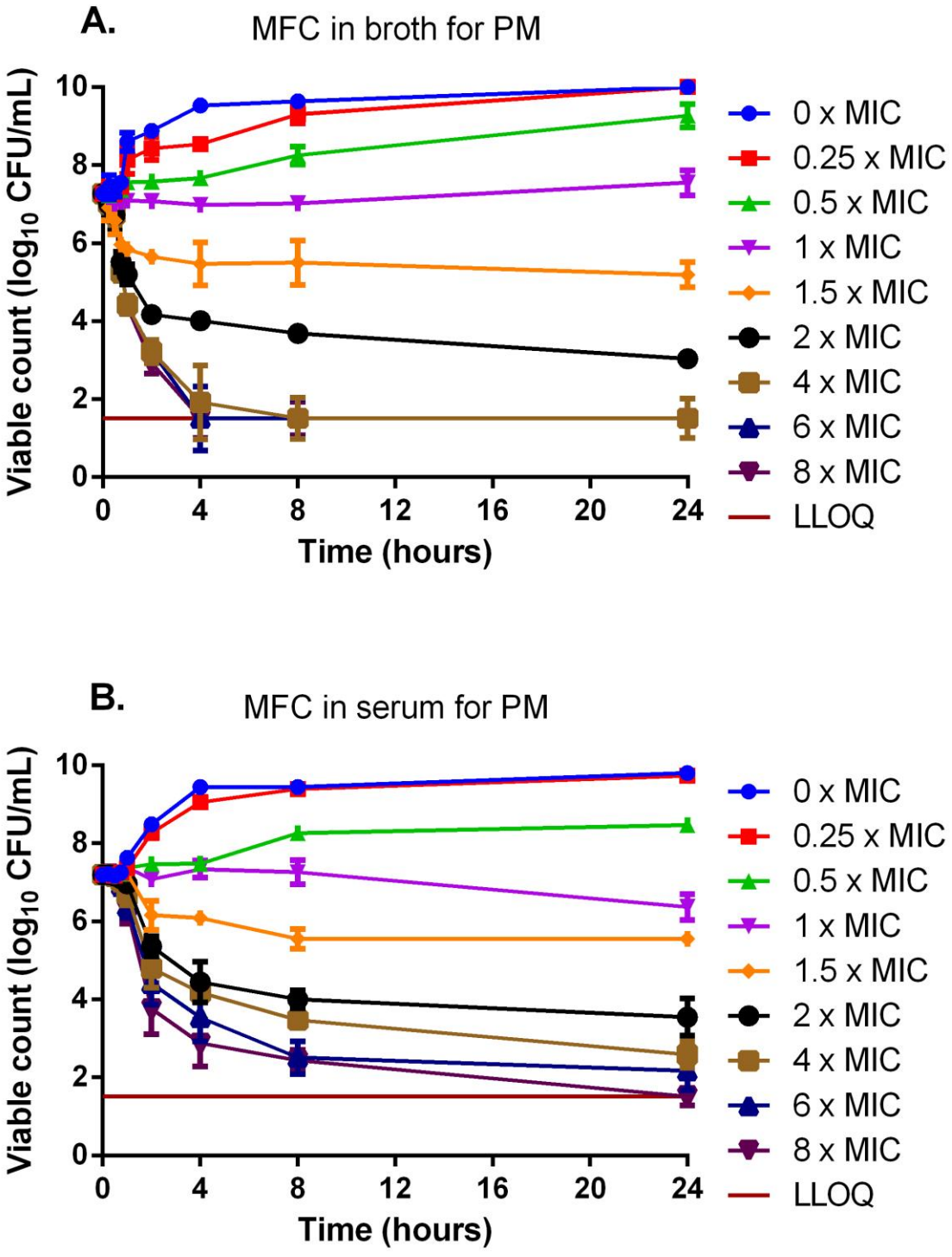

Figure 4

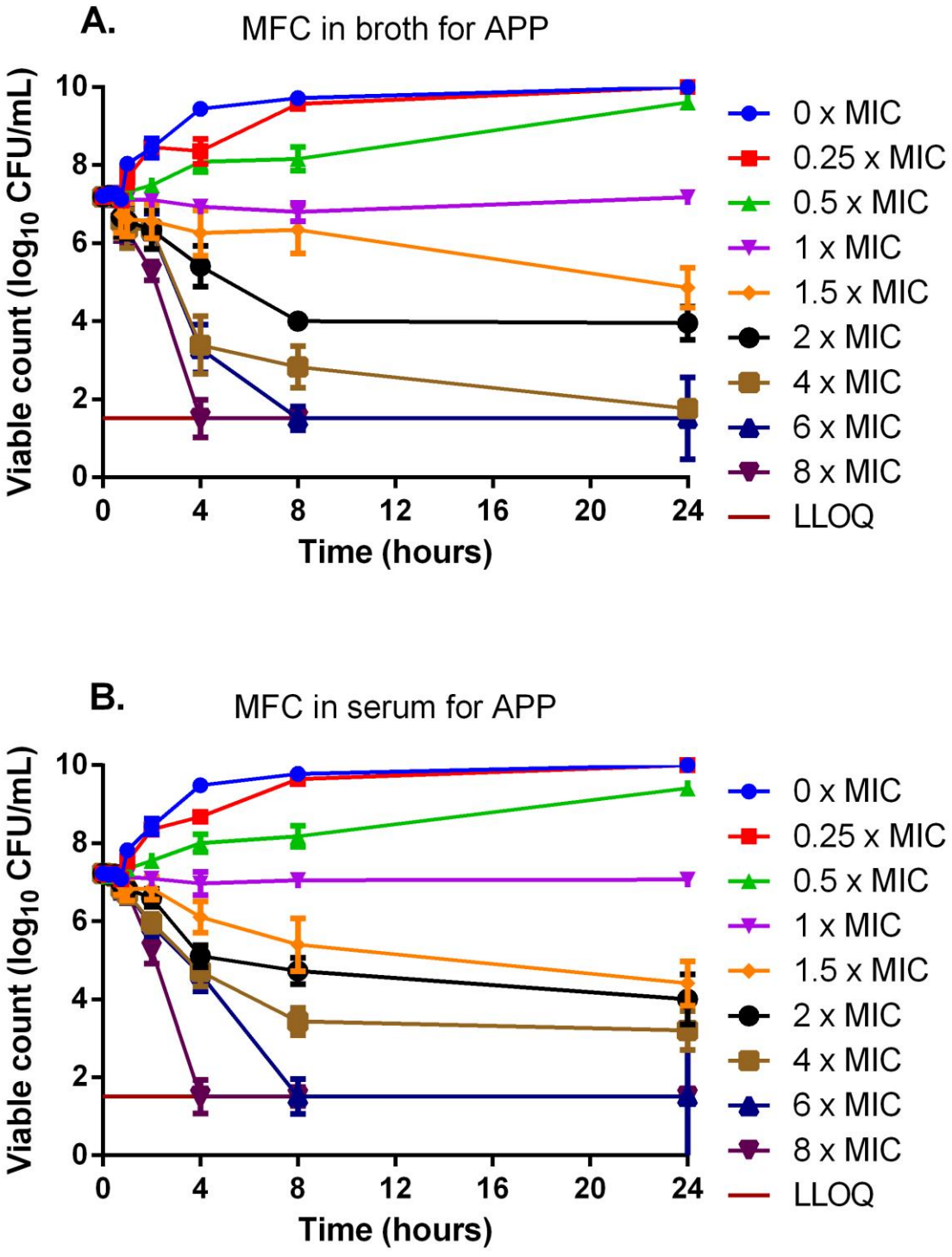

Figure 5

Part 2: Highlights

- Minimum Inhibitory Concentration of marbofloxacin was both inoculum size and growth matrix dependent (broth/serum comparison) for the pig pathogens, *P. multocida* and *A. pleuropneumoniae*
- Time-kill curves in broth and serum were concentration-dependent.