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A large potentiation effect of serum on the in vitro potency of tulathromycin against *Mannheimia haemolytica* and *Pasteurella multocida*

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Abstract

The antimicrobial properties of tulathromycin were investigated for M. haemolytica and P. multocida. Three in vitro indices of antimicrobial activity, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and time-kill curves, were established for six isolates of each organism. Each index was measured in two growth media: Mueller–Hinton broth (MHB) and calf serum. It was shown that MICs and MBCs were markedly lower in serum than in MHB. MHB:serum ratios for MIC were 47:1 (M. haemolytica) and 53:1 (P. multocida). For both serum and MHB, adjustment of pH led to greater potency at alkaline compared to acid pH. Tulathromycin MIC was influenced by size of inoculum count, being 4.0- to 7.7-fold greater for high compared to low initial counts. It was concluded that for the purpose of determining dosages for therapeutic use, pharmacodynamic data for tulathromycin should be derived in biological fluids such as serum. It is hypothesized that in vitro measurement of MIC in broth, conducted according to internationally recommended standards, may be misleading as a basis for estimating the in vivo potency of tulathromycin.

INTRODUCTION

Tulathromycin is a broad spectrum antimicrobial drug. Its spectrum of activity includes aerobic and anaerobic gram-positive bacteria, gram-negative cocci and Mannheima, Pasteurella, Actinobacillus, Haemophilus, Bordetella and Helicobacter, as well as mycoplasma, chlamydia and rickettsia species (Evans, 2005; Godinho et al., 2005a,b; Alexander et al., 2013; Villarino et al., 2013). As with other macrolides and triamilides, tulathromycin binds to the 50S ribosomal subunit, leading to blockade of transpeptidation and translocation reactions, to inhibit protein synthesis and hence prevent cell growth (Evans, 2005; Andersen et al., 2012). It is of interest that in 2004 percentage of bovine isolates of Mannheima haemolytica from USA and Canada with MICs of 16, 32, 64 and >64 μg/mL were 0, 0.6, 1.5 and 0.3, respectively. Corresponding percentage values in 2009 were 13.5, 3.3, 1.0 and 7.9, indicating reduction in potency for some isolates over this 5-year period. Similar findings were obtained for Pasteurella multocida (Portis et al., 2012).

Several authors have reported on the efficacy of tulathromycin in calves, as a prophylactic and metaphylactic drug for the prevention of pneumonia, and also at therapeutic dosages to treat calves with clinical signs of disease (Godinho et al., 2005a,b; Robb et al., 2007; Schunicht et al., 2007; Step et al., 2007; Nickell et al., 2008; Perrett et al., 2008; Van Donkersgoed et al., 2008, 2009; Rerat et al., 2012; Torres et al., 2013). In addition, Wellman and O'Connor (Wellman & O'Connor, 2007) undertook by meta-analysis a review of the then available clinical literature on tulathromycin, based on 21 manuscripts; comparing tulathromycin with tilmicosin, the summary Mantel–Haenszel relative risk for re-treatment of bovine respiratory disease was 0.51 (95% confidence interval 0.45–0.57), that is an approximately 50% reduction in risk for tulathromycin compared to tilmicosin.

Plasma concentrations of tulathromycin are consistently reported as being lower than in vitro MICs. Consequently, the classical PK/PD indices, based on in vitro MICs measured in broth, such as AUC/MIC, the appropriate index for long-acting macrolides, have been claimed to have no value in optimizing dosages. For example, the typical maximal plasma concentration of tulathromycin after subcutaneous administration at the recommended dosage (2.5 mg/kg) is approximately 500 pg/mL (Nowakowski et al., 2004) and this is considerably less than the typical MIC90s of calf isolates of M. haemolytica and P. multocida (Evans, 2005). It is clear that the CLSI/EUCAST methods to measure the in vitro potency of triamilide antimicrobial drugs, using Mueller–Hinton broth (MHB) as growth medium, are less than optimal when the

aim is to estimate numerical values of PK/PD indices. The aim of the present investigation was to measure the in vitro potency and efficacy of tulathromycin, a semi-synthetic drug of the triamilide class, in serum, that is in the relevant biological matrix to consider when in vitro generated data are to be directly incorporated in a PK/PD index and beyond, to compute an in vivo dosage regimen.

The objectives of this investigation were to: (i) compare the MIC and MBC of tulathromycin in two growth matrices, MHB and calf serum, for six clinical isolates of each of two bacterial species, M. haemolytica and P. multocida, harvested from cases of calf pneumonia; (ii) determine MICs for M. haemolytica and P. multocida in varying proportions of serum admixed with MHB; (iii) establish the effect of high, intermediate and low initial inoculum counts on MHB MICs; (iv) determine the effect of prior heat treatment of serum on serum MICs; (v) evaluate the effect on MICs of altering the concentrations of constituents of MHB to simulate their concentrations in serum; (vi) investigate the effect of varying matrix pH on MICs in serum and MHB; (vii) monitor the changes in pH produced by bacterial growth in MHB and serum in the absence of tulathromycin; (viii) determine time-kill profiles for six isolates of each species in serum and MHB, using five multiples of MIC, as a basis for classifying the killing action of tulathromycin as concentration-, time- or co-dependent.

MATERIALS AND METHODS

Origin, storage and selection of bacterial isolates

Twenty isolates of each of two calf pneumonia pathogens, M. haemolytica and P. multocida, were obtained at post mortem from field cases in several geographical regions of the United Kingdom. Each was supplied on swabs by the Animal Health and Veterinary Laboratories Agency (VLA, Addlestone, Surrey, U.K.) and stored at −70 °C in a fluid of composition glycerol:milk:water in the proportions 20:10:70. To sterilize, the fluid was boiled for 5 sec, allowed to cool for 12 h and then boiled again for a further 5 sec (Tyndall, 1877).

From the 20 isolates of each species, six of each species were selected for further study using two criteria: (i) each isolate was investigated for ability to grow logarithmically in two fluids, MHB and calf serum; (ii) each isolate was evaluated for sensitivity to tulathromycin using CLSI methods (CLSI, 2008), involving disc diffusion and measurement of diameter of zone of

growth inhibition. Sensitivity was then confirmed by determination of MICs, using doubling dilutions (CLSI, 2008). This initial screen ensured that all selected isolates could be used for MIC, MBC and time-kill determinations in the two growth matrices.

Culture methods and bacterial counts

Bacterial isolates were cultured in MHB (Oxoid, Loughborough, UK) or on 7% Sheep Blood agar [defibrinated sheep blood (TCS Biosciences, Buckingham, Bucks., UK) and blood agar base No. 2 (Oxoid)]. Agar cultures were incubated statically in a CO_2 incubator (Heraeus, Thermo Scientific, MA, USA) and MHB cultures were incubated with shaking at 150 rpm in a C25 incubating shaker (New Brunswick Scientific, NJ, USA) both at 37°C.

Viability counts were determined by serial dilution and spot-plate counts. Ten or 100-fold dilutions were made in Phosphate Buffered Saline (PBS) and three 10 µL drops of each dilution were transferred to the agar surface and allowed to dry for 10 min before incubating at 37°C.

Determination of Minimum Inhibitory and Minimum Bacterial Concentrations

Routine determination of minimum inhibitory and minimum bactericidal concentrations

MICs for six isolates each of *M. haemolytica* and *P. multocida* were established in accordance with CLSI methods (CLSI, 2004, 2008) except that, to increase accuracy, five overlapping sets of doubling dilutions of amoxicillin were prepared, instead of the CLSI method, which uses one set of doubling dilutions. In addition, calf serum collected commercially from animals <2 years old (Gibco, Paisley, Scotland, UK) was also used to determine MICs and MBCs using CLSI methods, except again using five overlapping sets of doubling dilutions and serum in place of broth. For determination of MICs in broth, the quality control strain used was *S. aureus* ATTC9144. The potency of the tulathromycin supplied was 96.8%. The standard solution of tulathromycin of strength 1.28 mg/mL was prepared in 0.0015 m citrate buffer, with pH adjusted to 7.0 with 0.015 m citric acid or 0.02 m NaOH, as required, with continual stirring. The solution was equilibrated at 70 ± 2 °C for 90 ± 5 min, with swirling at approximately 10

min intervals. The solution was cooled to room temperature, and the final dilution to 100 mL was prepared with purified water (further details supplied on request).

A volume of 97.5 μL of each dilution was aliquoted into the well of a *U*-shaped 96-well plate. Four mL of MHB was inoculated with a few colonies of the strain to be tested and incubated at 37 °C until growth, assessed by visual comparison with 0.5 McFarland Standard, reached approximately 1×10^8 cfu/mL. A volume of 2.5 μL of a 1:10 dilution of the culture in PBS was inoculated into each well. The plate was sealed with film, and incubated statically at 37 °C for 20 h. Serial dilutions (1–10−8) were prepared and spot-plate counts carried out immediately after inoculations. For each 10 μL, the bacterial count (cfu/mL) was determined after incubation for 18 h. The original inoculum count was calculated by taking the mean count for each 10 μL volume and multiplying by 100 to give the cfu/mL. This value was multiplied by the dilution factor to determine the initial cfu/mL. Determinations were repeated at least three times to confirm the measurement.

MBCs in MHB and serum were determined by microdilution, according to CLSI methods (CLSI, 2004), using a single set of doubling dilutions. MBC was determined using the spot-plate technique to establish a 3log10 decrease in the inoculum count.

Additional studies of minimum inhibitory concentration (conducted using five overlapping sets of doubling dilutions)

a. For four isolates of each species, MICs were determined in serum and in MHB supplemented to contain 25, 50 and 75% serum.
b. *MIC* measurements in MHB were undertaken for single isolates of *M. haemolytica* and *P. multocida*, to compare values for low, intermediate and high initial inoculum counts. The culture was grown to 1.0 McFarland Standard and diluted to final counts of 2.5–2.9×10^4, 2.5–2.9×10^6 and 2.5–2.9×10^8 cfu/mL, respectively.

c. The composition of calf serum and MHB differs significantly in respect of protein and albumin concentrations and of electrolytes (Brentnall et al., 2012). Further *MIC* determinations were therefore undertaken for two isolates each of *M. haemolytica* and *P. multocida*, in which MHB was supplemented with calcium alone, magnesium alone or calcium plus magnesium by addition of the chloride salts (British Drug Houses, VWR International, Poole, Dorset, U.K.) to achieve concentrations of these ions similar to those present in calf serum. Thus, calcium and magnesium were adjusted to give final concentrations of 2.77 nM/L (calcium) and 0.92 nM/L (magnesium). MHB was also supplemented with bovine serum albumin to provide a final concentration (28.5 mg/mL), similar to that occurring in normal calf serum of 21–36 mg/mL (Radostits et al., 2002).

d. For single isolates of *M. haemolytica* and *P. multocida*, MIC was re-determined in heat-treated serum to inactivate components such as complement, which might interact with tulathromycin on bacterial growth. The serum was heated in a water bath at 56 °C for 30 min.

e. The influence of matrix pH on the activity of tulathromycin was studied by adjusting the pH of MHB with dilute hydrochloric acid (l M) or sodium hydroxide (1 M) to achieve six pH values over the approximate range 7.0 to 8.0. For each pH, *MIC* was determined for four isolates each of *M. haemolytica* and *P. multocida*. The experiment was repeated with HEPES buffer (0.05 M) to provide a buffering capacity, similar to serum.

f. The effect of storage of calf serum at −20 °C for periods up to 28 days on pH and accompanying effects on *MIC* were monitored for single strains of *M. haemolytica* and *P. multocida* at five time points.

Influence of bacterial growth on matrix pH

To investigate the effect of bacterial growth on matrix pH, the pH was measured in the starting inoculum of an overnight culture, as described for time-kill curves (vide infra) and again after 24-h incubation for both MHB and serum. For each matrix, the initial pH was adjusted to be either low (7.18 for MHB and 7.33 for serum) by addition of 1 m HCL or high (8.17 for MHB and 8.27 for serum) by addition of 1 m NaOH. For each of the four matrices, the pH decrease (increasing acidity) over 24 h was monitored for four isolates each of M. haemolytica and P. multocida.

In vitro antimicrobial growth (time-kill) curves

For six isolates each of *M. haemolytica* and *P. multocida*, inoculating cultures were prepared by adding 3–4 colonies of the isolate to 4 mL MHB, followed by incubation overnight. Fifty microlitres of this culture was diluted 1:50 in prewarmed, freshly prepared MHB and incubated statically at 37 °C for one hour. The culture was then centrifuged at 31 000 g for 2 min. The supernatant was discarded, and the cells re-suspended in 50 μL PBS. The viable cell counts (cfu/mL) were determined by serial dilution and spot-plate counts.

Tulathromycin concentrations corresponding to 0.25, 0.5, 1, 2 and 4 × multiples of *MIC* for each isolate were prepared in prewarmed MHB or calf serum. For control growth curves, each matrix containing no drug was used. Four microlitres of the prepared culture was used to inoculate the dilutions to provide a 400 μL final volume. The cultures were placed in an orbital shaking incubator at 37 °C for 24 h. Forty microlitres of each culture were sampled and the viable count (cfu/mL) determined by serial dilution and spot-plate counts after incubation for 1, 2, 4, 8 and 24 h. The lowest detectable count was 33 cfu/mL.

Statistical analyses

MIC and MBC data are presented as geometric means and SD. Differences in *MIC* and MBC values between MHB and serum were compared using the paired *t*-test or the nonparametric Wilcoxon test, depending on whether the data passed a normality test.

RESULTS

Selection of isolates

From the 40 available isolates (20 of each species), six of each species were selected for further study to satisfy two criteria. First, a requirement to grow logarithmically in the two fluids, MHB and calf serum: percentages of the isolates were 65 and 65 (*M. haemolytica*) and 90 and 75 (*P. multocida*), respectively. Second, those isolates of both species, which grew logarithmically in both matrices, were further required to be classified as susceptible to tulathromycin (*MIC* $\leq$ 4.0 μg/mL). Six isolates of each species were then selected for further study, comprising highest, lowest and four with intermediate MICs.

Minimum inhibitory and minimum bactericidal concentrations

In all experiments, except those comparing low, intermediate and high starting counts, the initial inoculum count ranged from 5×10^5 to 5×10^6 cfu/mL. MIC and MBC values in MHB and serum for individual isolates are illustrated in Fig. 1. Table 1 presents data as geometric mean *MIC* and MBC and as ratios MBC:*MIC* and also broth:serum ratios for both MIC and MBC. For both organisms, geometric mean MICs and MBCs were significantly different between serum and MHB; the MHB:serum MIC ratios were 47:1 (*M. haemolytica*) and 53:1 (*P. multocida*). Corresponding MHB:serum MBC ratios were 58.7:1 and 48.0:1. For both growth matrices, MBC:*MIC* ratios were relatively low, in the range 1.4:1–1.8:1.

Further *MIC* determinations were made to compare values in MHB, 100% serum and varying proportions of serum (25, 50 and 75%) admixed with MHB (Table 2). As little as 25% serum incorporated in MHB reduced MICs ten- and sixfold for *M. haemolytica* and *P. multocida*, respectively. The admixture of MHB with greater amounts of serum produced further but proportionally smaller decreases in *MIC*.

Tulathromycin MICs in MHB were additionally determined using low, medium and high initial inoculum counts. For M. haemolytica, initial counts were 2.5×10^4, 2.5×10^6 and 2.5×10^8 cfu/mL, respectively. Corresponding initial counts for P. multocida were 2.9×10^4, 2.9×10^6 and 2.9×10^8 cfu/mL. For *M. haemolytica*, *MIC* ratios, high:medium and high:low starting counts, were 2.7:1 and 4.0:1, respectively. Corresponding values for *P. multocida* were 3.8:1 and 7.5:1.

For single isolates of *M. haemolytica* and *P. multocida*, serum *MIC* was re-determined after heating serum. Heat treatment did not alter *MIC* for either organism (data not shown).

For two isolates each of *M. haemolytica* and *P. multocida*, *MIC* was determined in MHB and MHB supplemented separately with calcium ions, magnesium ions, calcium plus magnesium ions or albumin to achieve concentrations of these cations and albumin similar to those present in normal calf serum. The mean MHB MICs were 0.20 μg/mL (*M. haemolytica*) and 0.25 μg/mL (*P. multocida*). Mean MICs were identical for both organisms for all supplemented matrices (data not shown).

For four isolates each of *M. haemolytica* and *P. multocida*, MICs in MHB were determined after adjustment of pH to six values over the approximate range 7.0–8.0. Results for two typical isolates are illustrated in Fig. 2. MICs decreased with increase in pH for both organisms, the changes being somewhat greater for *M. haemolytica*.

Following freezer storage (−20 °C), the pH of re-warmed serum was increased by 0.45 units within 7 days (Fig. 3). Storage for periods of 14, 21 and 28 days produced no additional apparent increase in pH. The increase in alkalinity was accompanied by reductions in *MIC* for single isolates of *M. haemolytica* and *P. multocida* (Fig. 3).

Influence of bacterial growth on matrix pH

In both MHB and serum, the growth of *M. haemolytica* and *P. multocida* (four isolates each) in the absence of tulathromycin led to reductions in pH over a 24-h incubation period (Table 3). When the initial pH was slightly acid, the decreases of 0.45 and 0.36 units in serum (*M. haemolytica* and *P. multocida*, respectively) were smaller than the decreases, 1.11 and 1.13, respectively, in MHB. Decreases in pH for serum when starting pH was alkaline were 1.18 and 0.69; corresponding values for MHB were 1.01 and 1.26, respectively, for *M. haemolytica* and *P. multocida* (Table 3). Bacterial growth was thus associated with acid production in all circumstances, being greater in magnitude for MHB compared to serum in three of four instances. As tulathromycin *MIC* is sensitive to small pH changes (Fig. 3), it is possible that the generally greater degree of acidification during growth in MHB, which is nonbuffered, would exert a greater impact on *MIC* than in serum, which is buffered. To further investigate this, MHB was buffered with HEPES to provide six pH values in the range 7.0–8.0. For P. multocida, buffering reduced MICs up to pH 7.6, but for *M. haemolytica*, buffering reduced *MIC* only at pH 7.0 and 7.2 (Fig. 4).

Time-kill curves

Tulathromycin time-kill curves, for five multiples of MIC in the range 0.25 to 4.0, are illustrated in Fig. 5 (*M. haemolytica*) and Fig. 6 (P. multocida). For both organisms, 0.25 and 0.5 multiples produced, at most, slight growth inhibition. For *M. haemolytica*, there was a more rapid and greater reduction in count at 2× and 4 × MIC in MHB than in serum. Thus, at 4 ×

MIC, the initial count was reduced by approximately 5 log10 cfu/mL in MHB after only 4-h exposure and to the same degree after 8-h exposure to 2 × *MIC*. The slower killing pattern of tulathromycin in serum was indicated by a 3 log10 reduction at 8 h at 4 × *MIC* and by a 4.5 $\log_{10}$ cfu/mL decrease in count only after 24-h incubation at 4 × *MIC*.

For *P. multocida*, there was a somewhat slower rate of kill at all concentrations compared to that achieved with *M. haemolytica*. Even at 24 h, a reduction in count to the level of detection was not achieved in either matrix. Reductions in count at 24 h were 4 $\log_{10}$ and 3 $\log_{10}$ with exposure to 4 × *MIC* for MHB and serum, respectively. Moreover, in serum, there was some re-growth at 24 h, compared to the 8-h count at 4 × *MIC*.

DISCUSSION

The principal finding in the present investigation is that, for two calf pathogens, *MIC* determined in a biologically relevant matrix (serum) was markedly lower than the MICs measured in an artificial matrix (MHB). Moreover, these serum *in vitro* MICs are consistent with in vivo plasma concentrations measured after a therapeutic dose of tulathromycin. The serum but not the MHB data will therefore enable recourse to the classical PK/PD *AUC/MIC* index to predict dosage requirement for clinical efficacy (Toutain et al. 2016).

Selection of an optimal dose for antimicrobial drugs depends on appropriate assessment of both pharmacokinetic (clearance and bioavailability) and pharmacodynamic (that is antimicrobial) properties. If the methodology yields only an approximate value for any parameter, for example *MIC*, dosage cannot be determined accurately. Hence, in this study, MICs were established using five overlapping sets of doubling dilutions to improve accuracy that is to reduce maximal estimation error from approaching 100% to not >20%. In addition, pharmacodynamic parameters were compared between serum and a nonphysiological fluid, MHB, and the latter is designed to be optimal for bacteriological growth *in vitro*. Such artificial growth matrices

are not necessarily predictive of bacterial growth in physiological fluids, and therefore may be poorly reflective of antimicrobial drug activity in vivo. Consideration of these differences, between biological fluids and artificial growth media, led Nightingale and Murakawa (Nightingale & Murakawa, 2002) to recommend use of physiological fluids for studies of antimicrobial activity (*MIC* determination and time-kill curves), when the objective is to set dosing schedules that are optimal for bacterial kill *in vivo*.

In this study, MICs were significantly lower in serum than in MHB. Similar findings for tulathromycin, based on MHB supplemented (up to 50%) with serum rather than the 100% serum used in the present study, have been reported by previous workers (Reese et al., 2004; Evans, 2005; Godinho et al., 2005a,b; Godinho, 2008). For example, Godhino et al. (Godinho et al., 2005a,b) reported that, for broth supplemented with 40% serum, the MIC of *M. haemolytica* was 0.25 μg/mL, compared to 2.0 μg/mL in broth. A similar eightfold reduction in MIC was reported for *P. multocida*. In the present study, we obtained 12-fold and ninefold decreases in MIC for *M. haemolytica* and *P. multocida*, respectively, using 50:50 serum:MHB mixtures compared to MHB. Importantly, even greater decreases in *MIC* were obtained using 100% serum as the growth matrix.

Total protein and albumin concentrations in the calf serum used in this study were 60.9 and 37.6 g/L, respectively (Brentnall et al., 2012). Corresponding concentrations in MHB were much lower, 3.78 and 0.08 g/L, respectively. *In vivo*, most antimicrobial drugs bind to serum proteins, mainly to albumin, to varying degrees, and for tulathromycin, the reported percentage protein binding was 32–47% [Pfizer 2005, 40% reported by European Medicines Agency (EMA)]. However, in our laboratory, protein binding of tulathromycin was higher (mean = 82%, Lees et al. unpublished data). Assuming little or no significant protein binding of tulathromycin in MHB and assuming also this to be the only factor leading to differences between serum and MHB, an approximately fivefold *increase* in *MIC* in serum compared to

MHB would be predicted. This is because protein bound drugs have been reported to be microbiologically inactive (Craig & Ebert, 1989; Zeitlinger et al., 2004, 2008). The difference in *MIC* between MHB and serum actually obtained was of the order of 50-fold but in favour of greater potency in serum. Comparing tulathromycin MICs for a single strain of *Mycoplasma mycoides mycoides* Small Colony, Mitchell et al. (Mitchell et al., 2012) reported a 330-fold lower value for serum compared to an artificial growth medium.

An early study by Pruul & McDonald (Pruul & McDonald, 1992) demonstrated that, at fixed acid (7.2) and alkaline (8.0) pH values, azithromycin MICs for *Escherichia coli* and *Staphylococcus aureus* were reduced eightfold to 60-fold by the incorporation of 40% serum in the growth medium. Moreover, these reductions in *MIC* were maintained in serum depleted of specified antibody and in serum lacking complement activity, but MICs were not reduced by the addition of either albumin or globulins to the broth. More recently, Rose et al. (Rose et al., 2013) reported that, for the novel veterinary macrolide tildipirosin, the addition to broth of increasing amounts of serum (from 5 to 50%) progressively reduced the MIC for six bacterial species harvested from pigs. Interestingly, *MIC* ratios for pure broth:broth supplemented with 50% serum were highest for *S. aureus* (8-64:1) and least for *M. haemolytica* (2-4:1) and *P. multocida* (1–2:1). From these data and the present findings, it may be concluded that the marked potentiating effect of serum is a class property of some newer macrolides and that it is species and strain (or isolate) as well as drug dependent. Consequently, it is clear that the CLSI methods for determining MICs in artificial growth matrices should not be used for the purpose of proposing dosage regimens for clinical use. That said, it is acknowledged that serum is not the biophase for lung infections; the biophase is the extracellular fluids that are an ultrafiltrate of plasma. Hence, the determination of MICs in epithelial lining fluid would be of relevance to the prediction of optimal dosage regimens (Kiem & Schentag, 2008). Nevertheless, serum

MICs are likely to provide a reasonable surrogate for epithelial lining fluid and thus be far more predictive than MHB of growth conditions in the biophase.

The present study investigated possible causes of the differences in *MIC* between MHB and calf serum, largely with negative results. Thus, alteration of the composition of MHB to increase, separately, albumin, calcium, magnesium and calcium plus magnesium concentrations, to be similar to those in calf serum, failed, in all instances, to alter *MIC*. On the other hand, the addition of calf serum to MHB, to produce final mixtures containing 25, 50 and 75% serum, did lead to progressive reductions in *MIC*. However, given the lack of effect on *MIC* of adjusting the albumin concentration of MHB to be similar to that in calf serum, our data suggest that a serum factor, other than albumin, must account for the lower MICs in both serum and MHB/serum mixtures compared to MHB.

For use in this study, serum-resistant isolates, that is those that grew logarithmically in serum, were necessarily selected, rather than the wider range of isolates representative of the whole populations, some of which are known to be killed by serum constituents such as complement. Including such strains might theoretically have lowered the mean *MIC* in serum, as complement might act synergistically with triamilides to promote growth inhibition, at least of growing organisms. In practice, however, *MIC* could not be determined in serum for isolates which do not reproduce logarithmically in this medium. Mitchell et al. (Mitchell et al., 2013) reported for another triamilide, gamithromycin, and a single isolate of *Mycoplasma mycoides* strain B237, a 500-fold increase in *MIC* in heat-inactivated serum compared with normal serum; respective MICs were 0.06 and 0.00012 μg/mL. In contrast, for one isolate each of the two bacterial species used in the present study, the same level of heat treatment used by Mitchell et al. (Mitchell et al., 2013) did not alter serum MICs.

Tulathromycin is a weak organic base (Evans, 2005). An effect of matrix pH on *MIC* is therefore not surprising, based on the fact that the ratio of unionized to ionized molecules will increase logarithmically as matrix pH increases. This is a simple manifestation of the Henderson–Hasselbalch principle. The more lipid-soluble unionized molecules would be expected to readily cross bacterial cell membranes, whereas the poorly lipid-soluble ionized molecules may penetrate less readily to the site of action within the bacterial cell, so that extent of penetration may be favoured by a more alkaline pH of the growth matrix. Under physiological and pathophysiological conditions, the extremes of pH of extracellular fluids compatible with life are of the order of 7.0 and 7.8. Therefore, in this study, the effect of pH on *MIC* in MHB was determined at two pH extremes (7.0 and 8.0) and at four intermediate values. The regression lines for each bacterial species indicated greater potency (lower *MIC*) as pH increased. The influence of pH on serum *MIC* values was further demonstrated by the effect of freezing samples at −20 °C. Within 7 days of freezing, the pH of re-warmed serum was increased by 0.5 units, possibly due to limited breakdown of proteins, with only slight further increases on more prolonged storage. MICs were re-determined on re-warmed samples; MICs were significantly reduced in the more alkaline samples. Similar data for *Haemophilus somni* were reported by Reese et al. (Reese et al., 2004). In summary, matrix pH seems likely to be an important factor accounting for markedly lower MICs in calf serum compared to MHB. However, it is unlikely to be the sole factor; the change in MIC between extremes of pH (7.0 and 8.0) as shown in Fig. 2 was smaller than the approximately 50-fold differences between serum and MHB.

During the logarithmic phase of growth, micro-organisms are metabolically highly active and this may lead to lowering of matrix pH. For a drug such as tulathromycin, whose growth inhibiting action is highly pH dependent, this may be a contributory factor to differences in *MIC* between MHB and serum reported in this study. Indeed, serum has a buffering capacity

that is likely to limit the reduction in matrix pH during growth, whereas buffering in MHB is likely to be absent, based on its chemical composition.

For four isolates each of *M. haemolytica* and *P. multocida*, the reductions in pH over 24-h growth periods were quantified for change from initial pHs, which were either acid or alkaline. For MHB for the two bacterial species, mean reductions in pH were 1.11, 1.01, 1.13 and 1.26. For serum, corresponding pH decreases during growth were 0.45, 1.18, 0.36 and 0.69. Hence, with the exception of serum with a starting alkaline pH for *M. haemolytica*, decreases in matrix pH were greater in MHB. Accumulation of acid during growth may therefore have contributed to the experimentally determined *MIC* differences between serum and MHB.

The main *MIC* data in this study were established for an intermediate starting bacterial count. However, for high compared to low starting inoculum counts, *M. haemolytica MIC* was fourfold greater and *P. multocida MIC* was 7.5-fold higher. Likewise, Mitchell et al. (Mitchell et al., 2012) reported a fourfold increase in *MIC* for tulathromycin for a starting inoculum counts of 10^7 compared to a count of 10^4 cfu/mL for *Mycoplasma mycoides mycoides* Small Colony. These findings are likely to be very relevant to the killing actions of tulathromycin *in vivo*. Thus, pathogen load in clinical disease is likely to be an important factor in determining an optimal dosage regimen for tulathromycin. Kesteman et al. (Kesteman et al., 2009) demonstrated the importance of pathogen load for the fluoroquinolone, marbofloxacin, in a rat lung *Klebsiella pneumoniae* infection model. The *AUC/ MIC* ratio preventing the emergence of resistance was fourfold higher with a high compared to a low pathogen load. Moreover, resistance development to marbofloxacin was greater in an *Escherichia coli* infection mouse thigh model, when the mice were infected with a high, compared to a low, initial inoculum count (Ferran et al., 2009).

In conclusion, the present study clearly showed a 50-fold reduction of *MIC* for tulathromycin in serum compared to the routinely used MHB. As a consequence, the MHB should be questioned when establishing PK/PD relationships as a basis for dosage regimen determination as reported in our companion paper (Toutain et al. 2016).

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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.

Anonymous (2005) Freedom information summary. Original new animal drug application. Draxxin injectable solution (Tulathromycin). In Freedom Information Summary. Original New Animal Drug Application. Draxxin

Injectable Solution (Tulathromycin), Ed Administration, F.a.D. pp. 1–57. PFIZER.

Bosnar, M., Kelneric, Z., Munic, V., Erakovic, V. & Parnham, M.J. (2005) Cellular uptake and efflux of azithromycin, erythromycin, clarithromycin, telithromycin, and cethromycin. Antimicrobial Agents and Chemotherapy, 49, 2372–2377.

Committee for Medicinal Products for Human Use (CHMP) (2011) Guideline on bioanalytical method validation. In Guideline on Bioanalytical Method Validation, pp. 1–23. European Medicines Agency, London.

Committee for Medicinal Products for Veterinary Use (CVMP) (2003) Scientific discussion – Draxxin. In Scientific Discussion – Draxxin, pp. 1 –31. European Medicines Agency, London.

Evans, N.A. (2005) Tulathromycin: an overview of a new triamilide antibiotic for livestock respiratory disease. Veterinary Therapeutics: Research in Applied Veterinary Medicine, 6, 83–95.

Gladue, R.P., Bright, G.M., Isaacson, R.E. & Newborg, M.F. (1989) In vitro and in vivo uptake of azithromycin (CP-62,993) by phagocytic cells: possible mechanism of delivery and release at sites of infection. Antimicrobial Agents and Chemotherapy, 33, 277–282.

Godinho, K.S. (2008) Susceptibility testing of tulathromycin: interpretative breakpoints and susceptibility of field isolates. Veterinary Microbiology, 129, 426–432.

Godinho, K.S., Keane, S.G., Nanjiani, I.A., Benchaoui, H.A., Sunderland, S.J., Jones, M.A., Weatherley, A.J., Gootz, T.D. & Rowan, T.G. (2005) Minimum inhibitory concentrations of tulathromycin against respiratory bacterial pathogens isolated from clinical cases in European cattle and swine and variability arising from changes in in vitro methodology. Veterinary Therapeutics: Research in Applied Veterinary Medicine, 6, 113–121.

Higgins, A.J., Lees, P. & Wright, J.A. (1984) Tissue-cage model for the collection of inflammatory exudate in ponies. Research in Veterinary Science, 36, 284–289.

Huang, R.A., Letendre, L.T., Banav, N., Fischer, J. & Somerville, B. (2010) Pharmacokinetics of gamithromycin in cattle with comparison of plasma and lung tissue concentrations and plasma antibacterial activity. Journal of Veterinary Pharmacology and Therapeutics, 33, 227–237.

Karlsson, M.O. & Savic, R.M. (2007) Diagnosing model diagnostics. Clinical Pharmacology and Therapeutics, 82, 17–20.

Kiem, S. & Schentag, J.J. (2008) Interpretation of antibiotic concentration ratios measured in epithelial lining fluid. Antimicrobial Agents and Chemotherapy, 52, 24–36.

Lees, P., Aliabadi, F.S. & Toutain, P.L. (2004) PK-PD modelling: an alternative to dose titration studies for antimicrobial drug dosage selection. Journal of Regulatory Affairs, 15, 175–180.

Mandell, G.L. & Coleman, E. (2001) Uptake, transport, and delivery of antimicrobial agents by human polymorphonuclear neutrophils. Antimicrobial Agents and Chemotherapy, 45, 1794–1798.

Menge, M., Rose, M., Bohland, C., Zschiesche, E., Kilp, S., Metz, W., Allan, M., Ropke, R. & Nurnberger, M. (2012) Pharmacokinetics of tildipirosin in bovine plasma, lung tissue, and bronchial fluid (from live, nonanesthetized cattle). Journal of Veterinary Pharmacology and Therapeutics, 35, 550–559.

Mouton, J.W., Theuretzbacher, U., Craig, W.A., Tulkens, P.M., Derendorf, H. & Cars, O. (2008) Tissue concentrations: do we ever learn? Journal of Antimicrobial Chemotherapy, 61, 235–237.

Muller, M., dela Pena, A. & Derendorf, H. (2004) Issues in pharmacokinetics and pharmacodynamics of anti-infective agents: distribution in tissue. Antimicrobial Agents and Chemotherapy, 48, 1441–1453.

Nowakowski, M.A., Inskeep, P.B., Risk, J.E., Skogerboe, T.L., Benchaoui, H.A., Meinert, T.R., Sherington, J. & Sunderland, S.J. (2004) Pharmacokinetics and lung tissue concentrations of tulathromycin, a new triamilide antibiotic, in cattle. Veterinary Therapeutics: Research in Applied Veterinary Medicine, 5, 60–74.

Roof, C. (2011) Qualification and quantification of bacterial pathogen load in acute bovine respiratory disease cases. In Qualification and Quantification of Bacterial Pathogen Load in Acute Bovine Respiratory Disease Cases. Kansas State University, Manhattan, Kansas.

Sarasola, P., Jernigan, A.D., Walker, D.K., Castledine, J., Smith, D.G. & Rowan, T.G. (2002) Pharmacokinetics of selamectin following intravenous, oral and topical administration in cats and dogs. Journal of Veterinary Pharmacology and Therapeutics, 25, 265–272.

Schentag, J.J. (2000) Clinical pharmacology of the fluoroquinolones: studies in human dynamic/kinetic models. Clinical Infectious Diseases, 31(Suppl 2), S40–S44.

Scheuch, E., Spieker, J., Venner, M. & Siegmund, W. (2007) Quantitative determination of the macrolide antibiotic tulathromycin in plasma and broncho-alveolar cells of foals using tandem mass spectrometry. Journal of Chromatography B, Analytical Technologies in the Biomedical and Life Sciences, 850, 464–470.

Scorneaux, B. & Shryock, T.R. (1998) Intracellular accumulation, subcellular distribution and efflux of tilmicosin in swine phagocytes. Journal of Veterinary Pharmacology and Therapeutics, 21, 257–268.

Scorneaux, B. & Shryock, T.R. (1999) Intracellular accumulation, subcellular distribution, and efflux of tilmicosin in bovine mammary, blood, and lung cells. Journal of Dairy Science, 82, 1202–1212.

Sidhu, P., Shojaee Aliabadi, F., Andrews, M. & Lees, P. (2003) Tissue chamber model of acute inflammation in farm animal species. Research in Veterinary Science, 74, 67–77.

Thomas, J.K., Forrest, A., Bhavnani, S.M., Hyatt, J.M., Cheng, A., Ballow, C.H. & Schentag, J.J. (1998) Pharmacodynamic evaluation of factors associated with the development of bacterial resistance in acutely ill patients during therapy. Antimicrobial Agents and Chemotherapy, 42, 521–527.

Toutain, P.L. (2009) PK/PD approach for antibiotics: tissue or blood drug levels to predict antibiotic efficacy. In PK/PD Approach for Antibiotics: Tissue or Blood Drug Levels to Predict Antibiotic Efficacy. Eds Riviere,

J.E. & Fink Gremmels, J., pp. 19–21. Journal of Veterinary Pharmacology and Therapeutics, Leipzig, Germany.

Toutain, P.L. & Lees, P. (2004) Integration and modelling of pharmacokinetic and pharmacodynamic data to optimize dosage regimens in veterinary medicine. Journal of Veterinary Pharmacology and Therapeutics, 27, 467–477.

Toutain, P.L., del Castillo, J.R.E. & Bousquet-M elou, A. (2002) The pharmacokinetic-pharmacodynamic approach to a rational dosage regimen for antibiotics. Research in Veterinary Science, 73, 105–114.

Toutain, P.L., Bousquet-Melou, A. & Martinez, M. (2007) AUC/MIC: a PK/PD index for antibiotics with a time dimension or simply a dimensionless scoring factor? Journal of Antimicrobial Chemotherapy, 60, 1185–1188.

Villarino, N., Brown, S.A. & Martin-Jimenez, T. (2013a) The role of the macrolide tulathromycin in veterinary medicine. Veterinary Journal, 198, 352–357.

Villarino, N., Lesman, S., Fielder, A., Garcia-Tapia, D., Cox, S., Lucas, M., Robinson, J., Brown, S.A. & Martin-Jimenez, T. (2013b)

Pulmonary pharmacokinetics of tulathromycin in swine. Part 2: intra-airways compartments. Journal of Veterinary Pharmacology and Therapeutics, 36, 340–349.

Villarino, N., Lesman, S., Fielder, A., Garcia-Tapia, D., Cox, S., Lucas, M., Robinson, J., Brown, S.A. & Martin-Jimenez, T. (2013c) Pulmonary pharmacokinetics of tulathromycin in swine. Part I: lung homogenate in healthy pigs and pigs challenged intratracheally with

lipopolysaccharide of Escherichia coli. Journal of Veterinary Pharmacology and Therapeutics, 36, 329–339.

Villarino, N., Brown, S.A. & Martin-Jimenez, T. (2014) Understanding the pharmacokinetics of tulathromycin: a pulmonary perspective. Journal of Veterinary Pharmacology and Therapeutics, 37, 211–221.

Waag, T.A., Bradford, J.R., Lucas, M.J., Rooney, K.A., Nutsch, R.G., Lechtenberg, K.F. & Smothers, C.D. (2008) Duration of effectiveness of tulathromycin injectable solution in an Actino bacillus pleuropneumoniae respiratory-disease challenge model in swine. Journal of Swine Health and Production, 16, 126–130.

Table 1. Geometric mean (SD) MIC and MBC in MHB and serum for amoxicillin and MIC:MBC and MHB:serum ratios (n=6)

M. haemolytica

Matrix	MIC	MBC	MBC:MIC ratio
MHB	2.07 (0.99)	3.67 (0.82)	1.8:1
Serum	0.04 (0.01)*	0.06 (0.00)*	1.4:1
Serum:MHB ratio	47.2 :1*	58.7 :1*	

P. multocida

Matrix	MIC	MBC	MBC:MIC ratio
MHB	2.07 (1.34)	3.00 (1.10)	1.5:1
Serum	0.04 (0.01)*	0.06 (0.00)*	1.6:1
Serum:MHB ratio	53.0:1*	48.0:1*	

Comparison of MHB/serum differences: *$P \leq 0.01$.

Table 2. Tulathromycin MICs (μg/mL) (geometric mean and SD) for eight isolates (four *M. haemolytica*and four *P. multocida*) in MHB and 25%, 50%, 75% and 100% calf serum

		Serum percentrage in MHB			
	MHB	25%	50%	75%	Serum
M. haemolytica	1.3 (0.12)	0.13 (0.03)[a]	0.11 (0.01)[a]	0.10 (0.02)[a]	0.07 (0.01)[a]
P. multocida	0.63 (0.10)	0.10 (0.02)[a]	0.07 (0.02)[a]	0.06 (0.04)[a]	0.05 (0.02)[a]

a Significantly different from MIC in MHB ($P \leq 0.01$).

Table 3. pH decrease (geometric mean and SD, n = 4) for MHB A (low pH), MHB B (high pH), serum A (low pH) and serum B (high pH)

	MHB A	MHB B	Serum A	Serum B
Starting pH	7.18	8.17	7.33	8.27
Reduction of pH for *M. haemolytica*	1.11 (0.1)	1.01 (0.17)	0.45 (0.15)[b]	1.18 (0.35)[a]
Reduction of pH for *P. multocida*	1.13 (0.07)[b]	1.26 (0.17)[a]	0.36 (0.09)[a,b]	0.69 (0.41)[a,b]

a pH decrease significantly different from that of MHB A.
b pH decrease significantly different from that of MHB B.

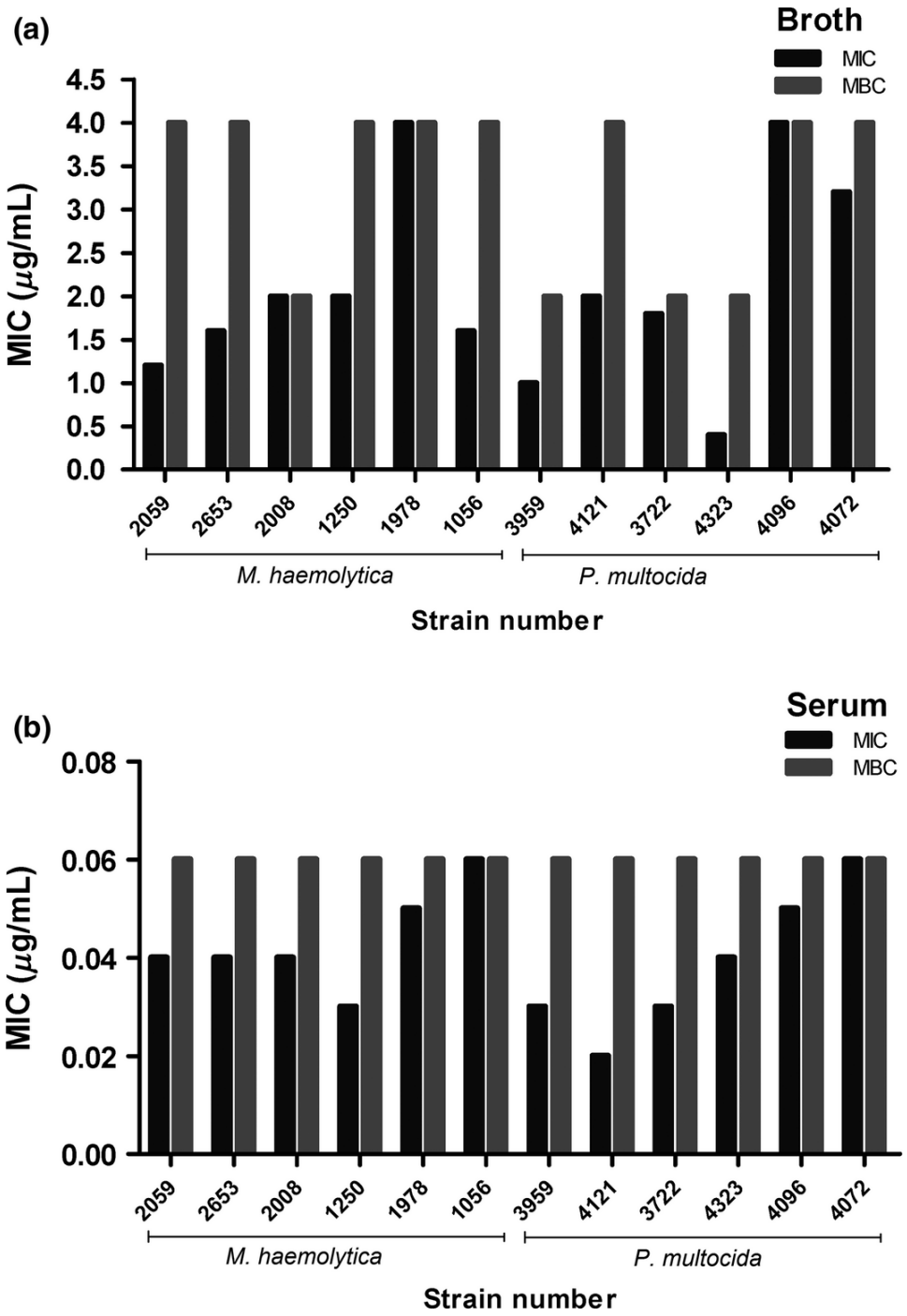

Fig. 1. MICs and MBCs of tulathromycin against 12 isolates (six *M. haemolytica*, first six left to right and six *P. multocida*, second six left to right) in MHB (a) and bovine serum (b).

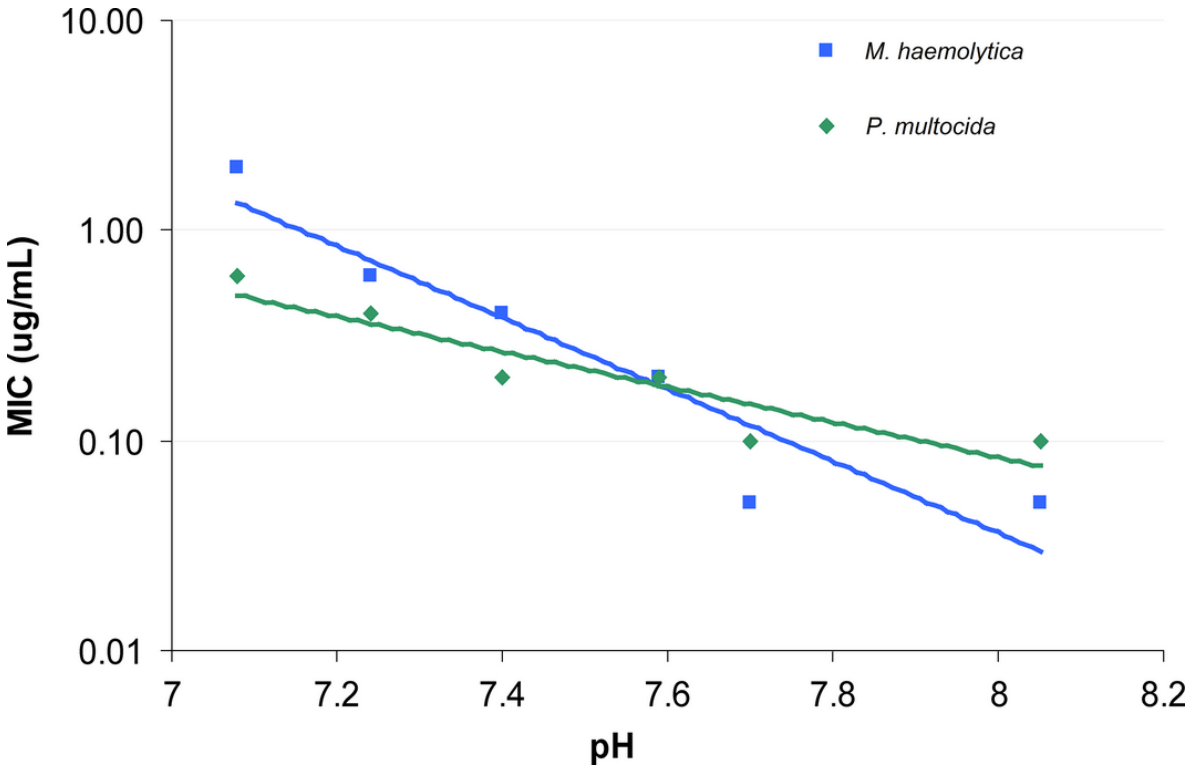

Fig. 2. Regression lines for *MIC*s of tulathromycin determined in MHB adjusted to six pH values

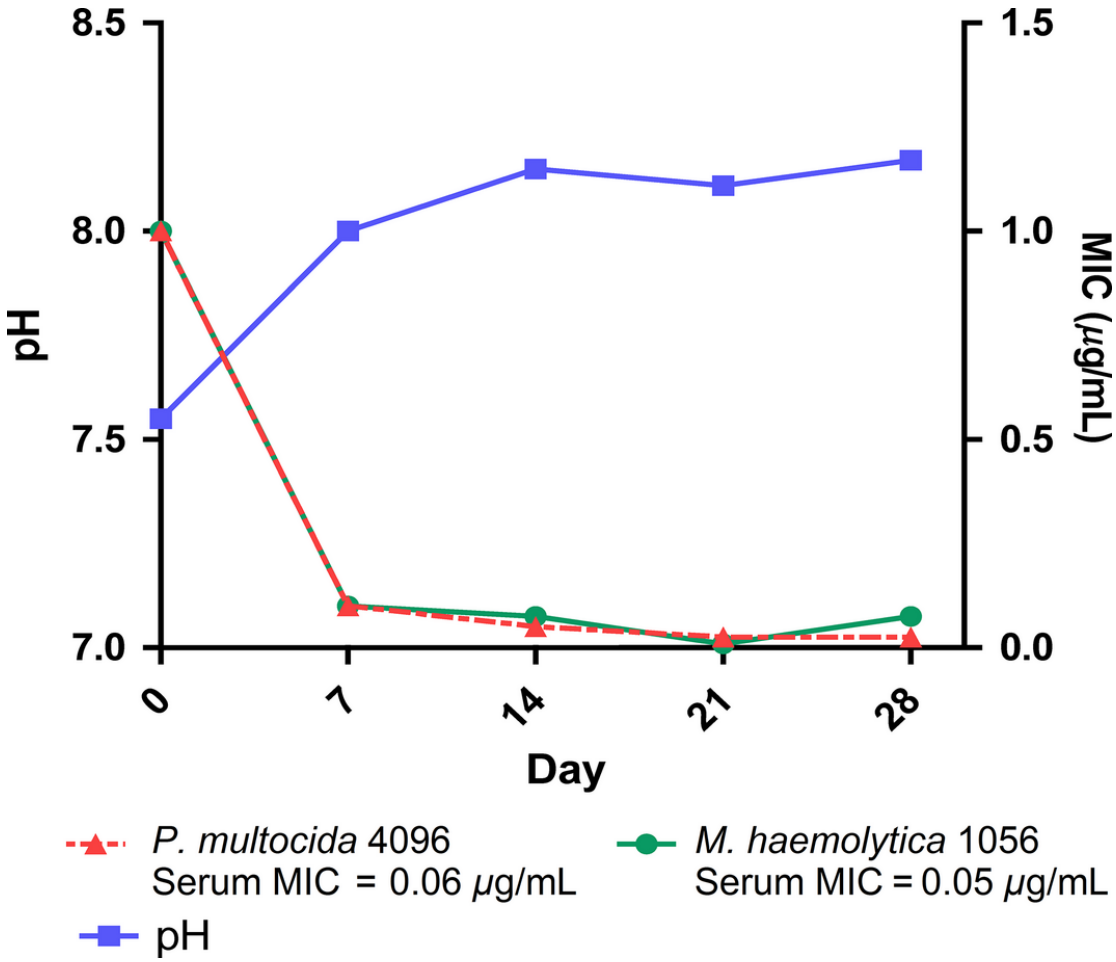

Fig. 3. *In vitro* inhibition of growth of *P. multocida* over 24 h exposure to five MIC multiples of amoxicillin in (a) MHB and (b) serum (mean, n=4). SEM bars not included for clarity.

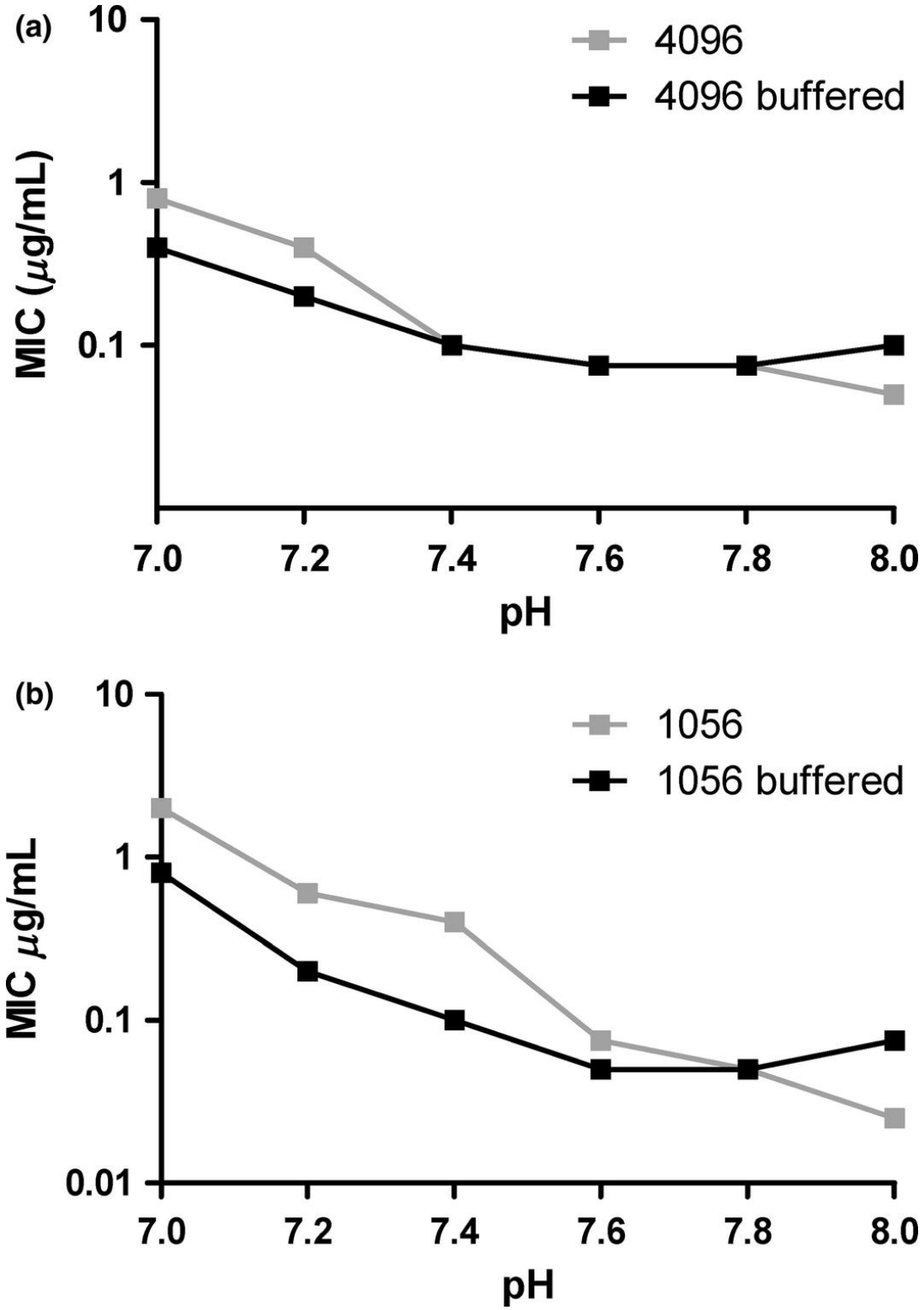

Fig. 4. *MIC*s for single isolates of *M. haemolytica* (a) and *P. multocida* (b) in MHB and buffered MHB at six pH values ranging from 7.0 to 8.0.

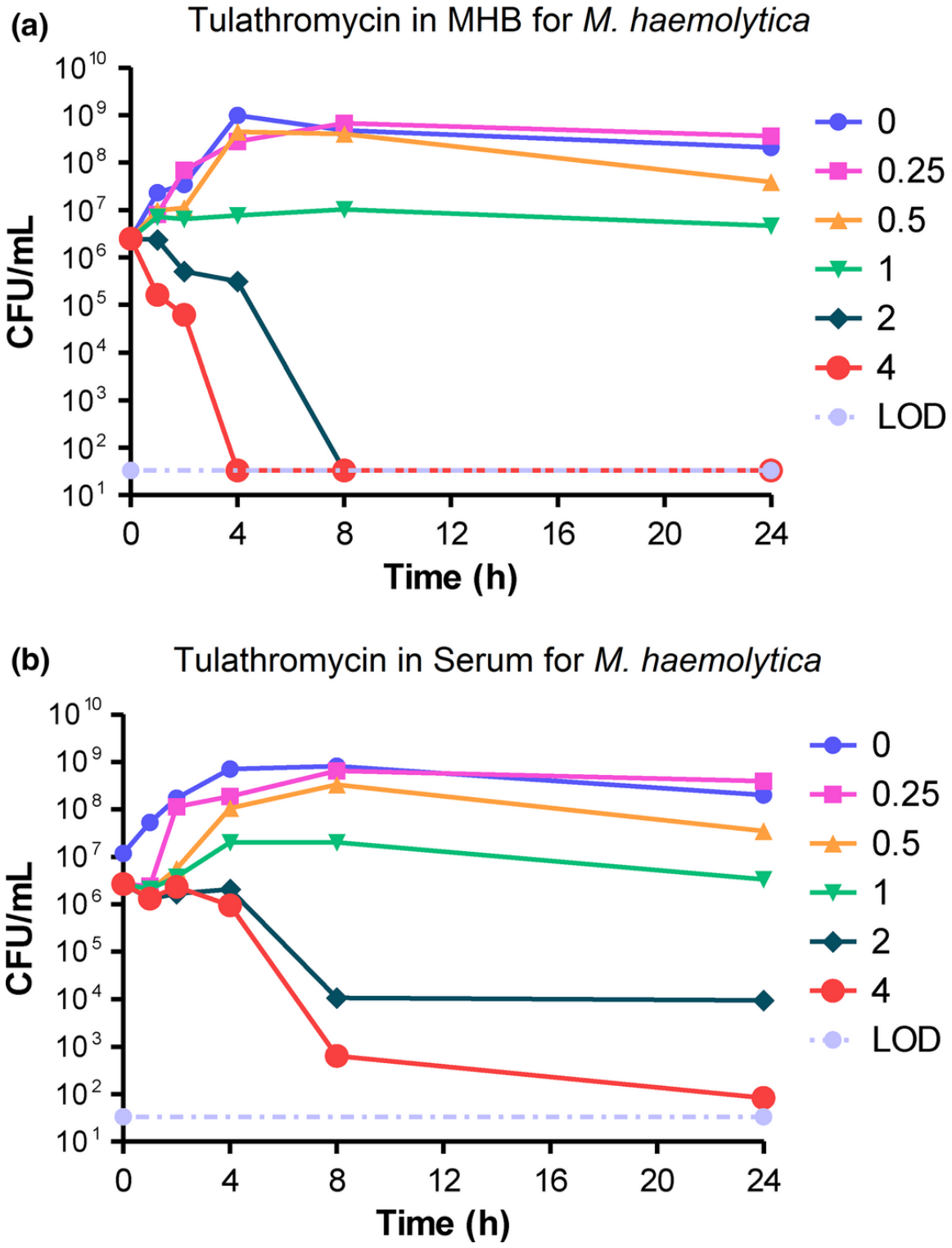

Fig. 5. *In vitro* inhibition of growth of *M. haemolytica* over 24-h exposure to five *MIC* multiples of tulathromycin in MHB (a) and serum (b) (geometric mean, *n* = 6 isolates, SEM bars not included for clarity).

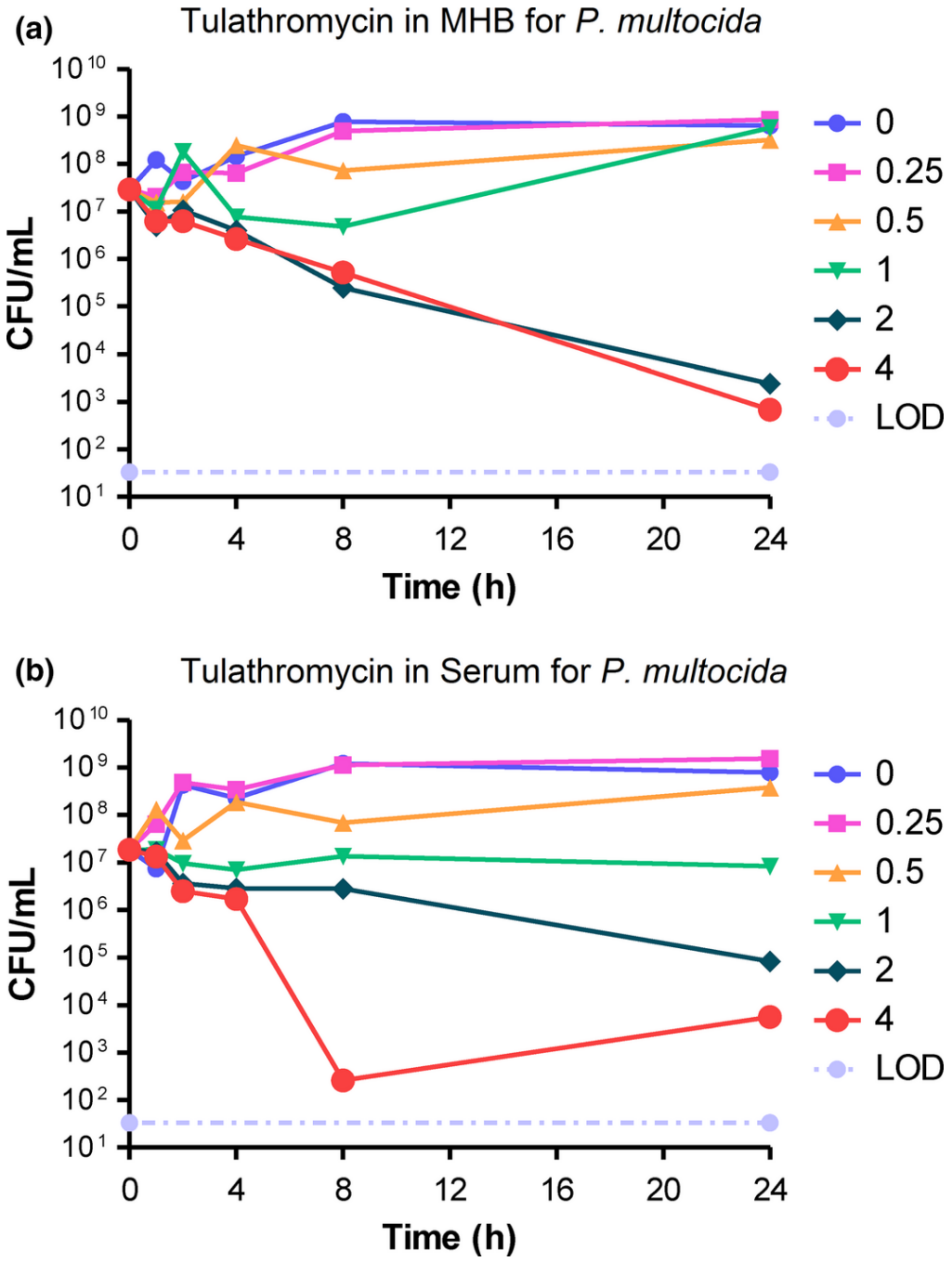

Fig 6. *In vitro* inhibition of growth of *P. multocida* over 24-h exposure to five multiples of *MIC* of tulathromycin in MHB (a) and serum (b) (mean, n = 6 isolates, SEM bars not included for clarity).