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Comparison of standardised versus non-standardised methods for testing the in vitro potency of oxytetracycline against Mannheimia haemolytica and Pasteurella multocida

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Highlights

- *Mannheimia haemolytica* and *Pasteurella multocida* isolates were obtained from cattle with respiratory disease.
- The pharmacodynamics of oxytetracycline were determined for *M. haemolytica* and *P. multocida*.
- Minimum inhibitory concentrations were substantially higher in serum than in broth.
- Serum broth differences were not attributable to protein binding.
- The clinical efficacy of oxytetracycline may not depend solely on killing bacteria.

Abstract

The in vitro pharmacodynamics of oxytetracycline were established for six isolates of each of the calf pneumonia pathogens *Mannheimia haemolytica* and *Pasteurella multocida*. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and bacterial time-kill curves were determined in two matrices, Mueller Hinton broth (MHB) and calf serum. Geometric mean MIC ratios, serum:MHB, were 25.2:1 (*M. haemolytica*) and 27.4:1 (*P. multocida*). The degree of binding of oxytetracycline to serum protein was 52.4%. Differences between serum and broth MICs could not be accounted for by oxytetracycline binding to serum protein. In vitro time-kill data suggested a co-dependent killing action of oxytetracycline. The in vitro data indicate inhibition of the killing action of oxytetracycline by serum factor(s). The nature of the inhibition requires further study. The outcome of treatment with oxytetracycline of respiratory tract infections in calves caused by *M. haemolytica* and *P. multocida* may not be related solely to a direct killing action.

Keywords: *Mannheimia haemolytica*; *Pasteurella multocida*; Bovine; Oxytetracycline; Pharmacodynamics
Introduction

Oxytetracycline is an antimicrobial drug with a broad spectrum of activity. The spectrum includes two bacterial pathogens implicated in bovine pneumonia, *Mannheimia haemolytica* and *Pasteurella multocida* (Nouws and Vree, 1983; Nouws et al., 1985; Nouws et al., 1990; Esaki et al., 2005). Oxytetracycline is still used extensively, despite the development of resistance in some species of bacteria. It is available in long acting, high strength formulations. These depot formulations provide sustained absorption from the site of injection (Nouws and Vree, 1983; Toutain and Raynaud, 1983; Davey et al., 1985; Nouws et al., 1990).

The potency of antimicrobial drugs is generally determined in vitro, based on the minimum inhibitory concentration (MIC). The widely accepted standards for MIC determination have been defined by the European Union Committee on Antimicrobial Sensitivity testing (EUCAST) and the Clinical Laboratory Standards Institute (CLSI); they ensure reproducible findings and thereby enable data to be harmonised internationally. This is essential when comparing data from several laboratories, between countries and across time periods for susceptibility testing. The two-fold dilution used is important because, when plotted on a histogram, the distributions are log-normal when using a log$_2$ distribution. The plots of histograms are more easily examined for the purpose of identifying wild-type distributions.

However, the standardised CLSI/EUCAST methods of determining MIC have two drawbacks for the purposes of this study. Firstly, they are based on two-fold, dilutions, with the potential consequence of up to 100% error, thus having a limitation regarding accuracy on single isolate estimates. Accuracy rather than precision is of importance in generating MIC...
data for the purpose of using pharmacodynamic (PD) data together with pharmacokinetic (PK) data for the purpose of dose prediction. To meet (in part) this concern, previous studies have used five sets of overlapping doubling dilutions to reduce inaccuracy on single isolate estimates (Aliabadi and Lees, 2001; Sidhu et al., 2010). Secondly, CLSI/EUCAST standards for MIC determinations are based on the use of artificial growth media. Whilst these provide optimal growth conditions in vitro, they differ in composition from biological fluids.

To provide comparisons between broths and biological fluids, previous studies have been undertaken in calf serum and inflammatory exudates (Aliabadi and Lees, 2001, 2002; Sidhu et al., 2003; Sidhu et al., 2010). A *M. haemolytica* isolate of calf origin had a MIC in serum 19 times greater than the broth MIC (Brentnall et al., 2012). Hence, the quantitative determination of PD indices with improved accuracy and in biological matrices, for some drug classes, may be helpful to the application of PK/PD approaches to dose determination. It is accepted that the vast majority of the published literature has relied on either EUCAST or CLSI methodology for determining MIC. This article therefore extends those findings.

Some authors recommend restriction of the term MIC for measurements undertaken in broths recommended by CLSI and EUCAST, with the requisite fluid being defined for each pathogenic species. However, the term MIC has been accepted in the peer reviewed literature, for other growth matrices and is retained in this article (Honeyman et al., 2015).

The aim of this study was to evaluate factors influencing the antimicrobial PDs of oxytetracycline for two calf pneumonia pathogens, *M. haemolytica* and *P. multocida*. The objectives were: (1) to compare in vitro MIC, MBC and time-kill profiles of oxytetracycline in two matrices, Mueller Hinton Broth (MHB) and calf serum; (2) to investigate the influence
of serum on MHB MICs by combining the two matrices in varying proportions; (3) to
determine the effect of low, intermediate and high bacterial counts on oxytetracycline MICs;
(4) to compare in vitro time-kill curves for oxytetracycline in MHB and calf serum; and (5) to
determine the degree of binding of oxytetracycline to protein in calf serum.

Materials and methods

Origin, storage, selection and culture of bacterial isolates

Twenty isolates of each of two calf pathogens, *M. haemolytica* and *P. multocida*, were
obtained post-mortem from field cases of calf pneumonia in various geographical regions of
the United Kingdom. They were supplied on swabs by the Veterinary Laboratories Agency
(AHVL), now Animal and Plant Health Agency (APHA), and stored at -70 °C in
glycerol:milk:water (20:10:70). This fluid was boiled for 5 s, left to cool for 12 h and then
boiled again for a further 5 s.

Two criteria were used to select 6/20 isolates of each of the two bacterial species for
further study: (1) each isolate was investigated for its ability to grow logarithmically in four
fluids (MHB and calf serum, exudate and transudate); (2) each isolate was evaluated for
susceptibility to oxytetracycline by disc diffusion and measurement of diameter of zone of
growth inhibition. Since tetracycline but not oxytetracycline is listed in the CLSI standards,
this measurement was not determined according to CLSI (2008). MICs were then determined
in MHB, using doubling dilutions.

Culture methods and bacterial viability counts, determined by serial dilution and spot-
plate counts, were as described by Lees et al. (2015).
Minimum inhibitory and minimum bactericidal concentrations

MICs for six isolates each of *M. haemolytica* and *P. multocida* were determined by broth microdilution in accordance with CLSI methods (CLSI, 2008), except that: (1) our study used MHB whereas CLSI requires use of cation adjusted MHB and, in future studies, the former would be preferred; and (2) to improve accuracy, five overlapping sets of doubling/two-fold dilutions of oxytetracycline were prepared in MHB, instead of the CLSI standard, which uses one set of doubling dilutions. Quality control (QC) organisms were not tested in this study to validate the assay because, for a small number of isolates, six of each of two species (as opposed to testing hundreds/thousands of isolates in constructing MIC distributions), this works well. What mattered more for this study was to reduce the error on individual estimates for a small number of isolates (12) from up to 100% to no more than 20%. This we did on the advice of A. Rycroft, Royal Veterinary College, University of London, United Kingdom).

The methods for MIC and MBC determinations were as previously described (Lees et al., 2015). The bactericidal assay was performed according to methods that have not been standardised by CLSI or EUCAST. QC was not performed on the MIC tests conducted for this study on oxytetracycline, because CLSI and EUCAST methods are based on tetracycline and not oxytetracycline. MIC determinations for the six isolates of both bacterial species were repeated, using five sets of overlapping two-fold dilutions of oxytetracycline prepared in bovine serum (Gibco). The influence of serum/MHB mixtures and inoculum size on MIC was determined as described in Appendix A.

Antimicrobial growth (time-kill) curves
For six isolates each of *M. haemolytica* and *P. multocida*, in vitro time-kill curves were established using oxytetracycline concentrations corresponding to 0.25, 0.5, 1, 2 and 4x multiples of MIC in both MHB and calf serum, as previously described (Lees et al., 2015). The lowest quantifiable count was 33 colony forming units (CFU)/mL. Ex vivo analyses were performed as described in Appendix B.

**Serum protein binding of oxytetracycline**

The degree of binding of oxytetracycline to serum protein in vivo was determined in triplicate on pooled samples from 10 calves harvested from a tissue cage study for seven concentrations, ranging from 0.43 to 2.07 µg/mL. The total concentration was measured on each sample as described in Appendix B and binding to protein was determined on a second aliquot of each sample by ultracentrifugation at 4,000 g and 25 °C for 20 min. The ultrafiltration device used was an Amicon Ultra Centrifugal filter (Ultracel 10 K, Millipore) and oxytetracycline concentrations were re-determined on the ultrafiltrate.

**Statistical analyses**

MIC and MBC data are presented as geometric means and standard deviation (SD). Differences in MIC and MBC values between MHB and serum were compared with a paired t test or the non-parametric Wilcoxon test, depending on whether the data passed a normality test.

**Results**

**Selection of isolates**

Six isolates of each species were selected to satisfy two criteria. Firstly, the percentages growing logarithmically were 65, 65, 40 and 55 for *M. haemolytica* and 90, 75,
65 and 65 for *P. multocida* for MHB, calf serum, exudate and transudate, respectively. Secondly, initial MIC studies using doubling dilutions indicated that the MIC for MHB was ≤ 0.4 µg/mL. It should be noted CLSI tables do not provide a separate breakpoint for oxytetracycline, but CLSI provides a breakpoint for tetracycline (≤ 2 µg/mL), and indicates that the breakpoint interpretation for tetracycline also applies to oxytetracycline. Therefore, the oxytetracycline MICs were less than the tetracycline breakpoint. The six isolates of each species selected comprised highest, lowest and four with intermediate MICs.

**Minimum inhibitory and minimum bactericidal concentrations**

MICs of the 12 selected isolates were re-determined separately in MHB and serum. MICs and MBCs are illustrated in Fig. 1. Table 1 presents geometric mean MICs and MBCs, and ratios MBC:MIC. The potency of oxytetracycline, expressed as MIC, was 25.2 times greater in MHB for *M. haemolytica* and 27.4 times greater in MHB for *P. multocida*, compared to serum MICs. Therefore, potency differed markedly between the two growth matrices. Using MBC as the indicator of potency indicated smaller differences than for MIC, but again in favour of MHB.

**Time-kill curves**

Starting inoculum counts of the order of 10<sup>7</sup> CFU/mL were selected to reflect a moderate to high bacterial load in clinical subjects (Roof, 2011). Despite marked differences in MICs between MHB and serum, growth inhibition curves in these matrices using multiples of MIC were broadly similar for *M. haemolytica* (Fig. 2). However, reductions in count were smaller in serum than in MHB at MIC multiples of 2.0 and 4.0. With both matrices, some re-growth occurred at 24 h. The in vitro killing pattern was classified as co-dependent (on both concentration and time). For *P. multocida* in MHB, MIC multiples of 2.0 and 4.0 produced
virtual eradication by 24 h (Fig. 3). In serum, the killing action at 24 h was less marked at 2x and 4x MIC than in MHB, with some re-growth occurring. The in vitro killing action was judged to be co-dependent for both matrices (Fig. 3). Ex vivo time-kill curves are shown in Appendix B.

**Binding to serum protein**

Oxytetracycline binding to protein was established for a range of seven in vivo concentrations (0.43 to 2.07 µg/mL) in pooled serum samples. Mean percentage protein binding ± SD was 52.4 ± 7.3 and was independent of total concentration.

**Discussion**

Many previous studies have shown that, for some drugs of the macrolide/triamilide groups, MICs determined in serum are much higher than those determined using the broths recommended in CLSI and EUCAST guidelines. An example is tulathromycin; for this drug Toutain et al. (2016) reported MICs some 50 times lower in bovine serum than in broths for *M. haemolytica* and *P. multocida* of bovine origin. When serum values were corrected for drug binding to serum protein, differences were even greater; causes have not been positively determined. In contrast, in this study the ‘serum effect’ was reversed; MICs were higher in broth than in serum. Toutain et al. (2016) showed clearly that accuracy of MIC determination, to be used in dose prediction, can be obtained from the approved broth MIC data by applying a robust scaling factor to bridge in vitro MHB to in vivo relevant serum/plasma/blood values. Oxytetracycline MICs were reported against a single bovine isolate of *M. haemolytica* (A1 76/1), in five matrices (Brentnall et al., 2012, 2013); values (µg/mL) were 0.5 (MHB), 0.8 (cation adjusted MHB), 14.8 (serum), 12.8 (exudate) and 11.2 (transudate). Therefore, MIC differed markedly between three biological fluids on the one hand and two artificial media on
the other, whilst similar values were obtained for the two artificial matrices and similar values were obtained for the three biological fluids.

These findings were confirmed and extended to demonstrate: (1) much higher MICs of oxytetracycline in serum compared to MHB for *M. haemolytica* and *P. multocida*; (2) higher oxytetracycline MBCs in serum compared to MHB, but with lower serum:MHB ratios for MBC than for MIC. This study also quantified inter- and intra-species differences in variability for six isolates of each species; coefficient of variation (CV%) serum values for *M. haemolytica* were 84 (MIC) and 62 (MBC). Corresponding CV% for *P. multocida* were lower (38 MIC; 33 MBC). Considering the serum MIC values reported in this study, and species and isolate variability, it is important for future studies to recognise that we used bovine serum from a single source. Breed, age, sex, disease state, country and other factors might provide MIC differences between sera, even from a single species. Such differences should be quantified. However, this paper had a more limited immediate goal, to compare broth with serum from the target species derived from one source only.

The cause(s) of serum/MHB differences in MIC for oxytetracycline have not been established. In a recent study, albumin concentrations in MHB and calf serum were 0.033 and 32.2 g/L, thus differing by approximately 1,000-fold (Brentnall et al., 2012). Most serum protein binding occurs to albumin and it is very likely that total and free concentrations of oxytetracycline in MHB were identical. Therefore, approximately two-fold higher MIC values in serum compared to MHB would be anticipated from the binding of oxytetracycline to serum protein, which was shown to be 52.4% of total concentration. This confirms the 50% binding described by Pilloud (1973) and is intermediate between 18.6% (Ziv and Sulman, 1972) and 72% (Nouws et al., 1985) described for cattle by other authors. Cause(s) of
differing degrees of protein binding in these studies are not known, but it should be noted that the degree of binding has an impact on dosages required to achieve a given level of efficacy. The prediction of approximately two-fold higher MICs in serum in the present study arises because protein bound AMDs are microbiologically inactive (Wise, 1986; Zeitlinger et al., 2004). This relatively small (two-fold) predicted difference is well short of the 25- to 27-fold experimentally determined differences in MIC between MHB and serum for *M. haemolytica* and *P. multocida*, respectively.

In quantitative terms, for *P. multocida*, correction for protein binding yields a mean fraction unbound (fu) serum MIC of 3.21 µg/mL, whilst broth MIC was 0.25 µg/mL; thus, the mean fu serum MIC is 12.9-fold greater than the broth MIC, which is the CLSI and EUCAST and therefore universally accepted standard. For *M. haemolytica*, the fu serum MIC was 11.8 times greater than broth MIC. The data indicate inhibition of the killing action of oxytetracycline by some serum factor(s). The data demonstrate antagonism of the action of oxytetracycline beyond what can be ascribed to non-specific protein binding.

The nature of the inhibition requires further consideration. In addition to albumin content, other differences in composition between MHB and calf serum include higher globulin, sodium, chloride, potassium, calcium and magnesium concentrations (Brentnall et al., 2012). Since oxytetracycline can bind covalently to calcium and magnesium ions, this might theoretically explain the serum/broth MIC and MBC differences. However, Luthman and Jacobsson (1983) reported that oxytetracycline did not chelate with calcium ions in calf serum. Moreover, the MIC difference between MHB and cation adjusted MHB reported by Brentnall et al. (2012) was slight (0.5 and 0.8 µg/mL, respectively). Therefore, the cause(s) of
the marked differences in MIC between MHB and cation adjusted MHB on the one hand and
calf serum on the other require alternative explanations and further study.

Honeyman et al. (2015) compared MICs of several tetracyclines in broth and 50% broth:50% mouse serum as matrices. For a strain of *Streptococcus pneumoniae*, MICs were identical for six compounds but, with added serum, 2-4 fold increases were obtained for five, whilst MIC was increased 32-fold for one compound. In contrast, for a strain of *Staphylococcus 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- to 128-fold.

The PDs of oxytetracycline was further investigated in time-kill studies. Using multiples of up to 4x MIC indicated a probable co-dependent killing mechanism that is dependency on both concentration and time. However, confirming the type of killing action would benefit from further studies using higher multiples of MIC than the five used in this study.

These data suggest that serum, exudate and transudate may be useful alternatives to broth for potency determination, when the objective is estimation of a dose for clinical use, based on PK/PD modelling approaches. These biological fluids are not identical to pulmonary epithelial lining fluid, but are much closer in composition to the latter than artificial broths. Further refinement of the methodology used in this study would be to determine potency in serum in the presence of other ‘natural’ constituents, such as leucocytes and antibodies, as well as the normal bacterial flora that compete with pathogens. Ideally, although technically difficult, it would also be relevant to determine potency in pulmonary epithelial lining fluid.
Despite these considerations, in immunocompetent animals with pneumonic infections, even the limited direct killing activity in serum demonstrated in this study might contribute to efficacy, particularly in those cases with mild infection, treated early, in which biophase bacterial counts would normally be low. Epidemiological data on oxytetracycline MICs have indicated a bimodal distribution (Yoshimura et al., 2001). Even allowing for these MICs, measured conventionally in an artificial growth matrix, some 40-50% of isolates had MICs of 8.0 or 16.0 µg/mL for calf strains of *M. haemolytica* and *P. multocida*. On the other hand, 30 to 40% of isolates had broth MICs of 0.50 µg/mL; the equivalent serum MIC, from the present data, would be of the order of 12.5 µg/mL, which is approximately two to three times higher than maximum serum concentrations of oxytetracycline achieved in calves with the recommended dose rate of 20 mg/kg (Nouws and Vree, 1983; Toutain and Raynaud, 1983; Nouws et al., 1990; Brentnall et al., 2013). Alternative mechanisms of action of oxytetracycline are shown in Appendix C.

**Conclusions**

Concentrations of oxytetracycline in serum and broth were not measured at the start and completion of the in vitro studies; it is possible that reported differences between the media might have been due, in part, to some degradation of the drug, but at differing rates over the 24 h incubation periods. Differing bacterial growth rates in the two media are possible, even likely, and this could contribute to the reported differences. Time-kill studies were based on fixed concentrations for a pre-defined time period. In vivo, concentrations in serum and the biophase first increase and then decrease after systemic, non-vascular dosing. Therefore, in vitro time-kill methods, such as hollow fibre models, better reflect the circumstances of clinical exposure and could be used in future studies.
Conflict of interest statement

None of the authors of this paper have a financial or personal relationship with other people or organisation that could inappropriately influence or bias the content of the paper. In the last 5 years, P. Lees has supplied consultancy advice to Bayer Animal Health, Norbrook Laboratories and Pfizer Animal Health, J. Illambas was formerly employed by Zoetis Animal Health and L. Pelligand provided consultancy advice to VetCare, Orion, Zoetis, Waltham and Ceva, as well as receiving research funding from Pfizer Animal Health, Novartis Animal Health, Transpharmation and deltaDot, and P.L. Toutain provided consultancy advice to Novartis Animal Health.

Acknowledgements

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Appendix: Supplementary Material

Supplementary data associated with this article can be found, in the online version, at doi: …

References


Roof, C., 2011. Qualification and quantification of bacterial pathogen load in acute bovine respiratory disease cases. Masters Thesis, Kansas State University, Manhattan, Kansas, USA.


Figure legends

Fig. 1. Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) for oxytetracycline against 12 isolates (first six, left to right *Mannheimia haemolytica*; second six, left to right *Pasteurella multocida*) in Mueller Hinton broth (MHB) and bovine serum. Note the differing ordinate scales.

Fig. 2. In vitro inhibition of growth of *Mannheimia haemolytica* over 24 h exposure to five multiples (0.25 to 4.0) of minimum inhibitory concentration (MIC), measured in either Mueller Hinton broth (MHB) or serum, for oxytetracycline: (a) MHB and (b) serum (mean for six isolates in each matrix). Standard error of the mean (SEM) bars not included for clarity. Dotted line indicates limit of quantification (33 colony forming units, CFU/mL).

Fig. 3. In vitro inhibition of growth of *Pasteurella multocida* over 24 h exposure to five minimum inhibitory concentrations (MIC) multiples (0.25 to 4.0) of oxytetracycline, measured in either Mueller Hinton broth (MHB) or serum: (a) MHB and (b) serum (mean for six isolates in each matrix). Standard error of the mean (SEM) bars not included for clarity. Dotted line indicates limit of quantification (33 colony forming units, CFU/mL).
Table 1. Oxytetracycline geometric mean standard deviation (SD) minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) measured in Mueller Hinton broth (MHB) and serum, along with MIC:MBC and MHB:serum ratios ($n = 6$).

<table>
<thead>
<tr>
<th>Matrix</th>
<th>MIC (µg/mL)</th>
<th>MBC (µg/mL)</th>
<th>MBC:MIC ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannheimia haemolytica</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MHB</td>
<td>0.22 (0.12)</td>
<td>2.83 (2.51)</td>
<td>13.1:1</td>
</tr>
<tr>
<td>Serum</td>
<td>5.46 (4.61)</td>
<td>10.08 (6.20)</td>
<td>1.9:1</td>
</tr>
<tr>
<td>Serum:MHB ratio</td>
<td>25.2:1</td>
<td>3.6:1</td>
<td></td>
</tr>
<tr>
<td>Pasteurella multocida</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MHB</td>
<td>0.25 (0.08)</td>
<td>1.26 (1.33)</td>
<td>5.1:1</td>
</tr>
<tr>
<td>Serum</td>
<td>6.75 (2.58)</td>
<td>12.67 (4.13)</td>
<td>1.9:1</td>
</tr>
<tr>
<td>Serum:MHB ratio</td>
<td>27.4:1</td>
<td>10.1:1</td>
<td></td>
</tr>
</tbody>
</table>

Significant difference between MHB and serum: $^a P < 0.05; ^b P < 0.01$. 
Figure 1.

**Broth**

**Serum**
Figure 2.

(a) Oxytetracycline in MHB for *M. haemolytica*

(b) Oxytetracycline in Serum for *M. haemolytica*
Figure 3.

**Oxytetracycline in MHB for *P. multocida***

- **a**
  - Graph showing CFU/mL over time (h) for different concentrations of Oxytetracycline.
  - Legend: 0, 0.25, 0.5, 1, 2, 4, LOD.

**Oxytetracycline in Serum for *P. multocida***

- **b**
  - Graph showing CFU/mL over time (h) for different concentrations of Oxytetracycline.
  - Legend: 0, 0.25, 0.5, 1, 2, 4, LOD.