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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

42 Introduction

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

51
 52 The potency of antimicrobial drugs is generally determined in vitro, based on the
 53 minimum inhibitory concentration (MIC). The widely accepted standards for MIC
 54 determination have been defined by the European Union Committee on Antimicrobial
 55 Sensitivity testing (EUCAST) and the Clinical Laboratory Standards Institute (CLSI); they
 56 ensure reproducible findings and thereby enable data to be harmonised internationally. This is
 57 essential when comparing data from several laboratories, between countries and across time
 58 periods for susceptibility testing. The two-fold dilution used is important because, when
 59 plotted on a histogram, the distributions are log-normal when using a \log_2 distribution. The
 60 plots of histograms are more easily examined for the purpose of identifying wild-type
 61 distributions.

62
 63 However, the standardised CLSI/EUCAST methods of determining MIC have two
 64 drawbacks for the purposes of this study. Firstly, they are based on two-fold, dilutions, with
 65 the potential consequence of up to 100% error, thus having a limitation regarding accuracy on
 66 single isolate estimates. Accuracy rather than precision is of importance in generating MIC

67 data for the purpose of using pharmacodynamic (PD) data together with pharmacokinetic
 68 (PK) data for the purpose of dose prediction. To meet (in part) this concern, previous studies
 69 have used five sets of overlapping doubling dilutions to reduce inaccuracy on single isolate
 70 estimates (Aliabadi and Lees, 2001; Sidhu et al., 2010). Secondly, CLSI/EUCAST standards
 71 for MIC determinations are based on the use of artificial growth media. Whilst these provide
 72 optimal growth conditions in vitro, they differ in composition from biological fluids.

73
 74 To provide comparisons between broths and biological fluids, previous studies have
 75 been undertaken in calf serum and inflammatory exudates (Aliabadi and Lees, 2001, 2002;
 76 Sidhu et al., 2003; Sidhu et al., 2010). A *M. haemolytica* isolate of calf origin had a MIC in
 77 serum 19 times greater than the broth MIC (Brentnall et al., 2012). Hence, the quantitative
 78 determination of PD indices with improved accuracy and in biological matrices, for some
 79 drug classes, may be helpful to the application of PK/PD approaches to dose determination. It
 80 is accepted that the vast majority of the published literature has relied on either EUCAST or
 81 CLSI methodology for determining MIC. This article therefore extends those findings.

82
 83 Some authors recommend restriction of the term MIC for measurements undertaken in
 84 broths recommended by CLSI and EUCAST, with the requisite fluid being defined for each
 85 pathogenic species. However, the term MIC has been accepted in the peer reviewed literature,
 86 for other growth matrices and is retained in this article (Honeyman et al., 2015).

87
 88 The aim of this study was to evaluate factors influencing the antimicrobial PDs of
 89 oxytetracycline for two calf pneumonia pathogens, *M. haemolytica* and *P. multocida*. The
 90 objectives were: (1) to compare in vitro MIC, MBC and time-kill profiles of oxytetracycline
 91 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 (AHVLA), 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).

117 *Minimum inhibitory and minimum bactericidal concentrations*

118 MICs for six isolates each of *M. haemolytica* and *P. multocida* were determined by
 119 broth microdilution in accordance with CLSI methods (CLSI, 2008), except that: (1) our study
 120 used MHB whereas CLSI requires use of cation adjusted MHB and, in future studies, the
 121 former would be preferred; and (2) to improve accuracy, five overlapping sets of
 122 doubling/two-fold dilutions of oxytetracycline were prepared in MHB, instead of the CLSI
 123 standard, which uses one set of doubling dilutions. Quality control (QC) organisms were not
 124 tested in this study to validate the assay because, for a small number of isolates, six of each of
 125 two species (as opposed to testing hundreds/thousands of isolates in constructing MIC
 126 distributions), this works well. What mattered more for this study was to reduce the error on
 127 individual estimates for a small number of isolates (12) from up to 100% to no more than
 128 20%. This we did on the advice of A. Rycroft, Royal Veterinary College, University of
 129 London, United Kingdom).

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

140 *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 \leq 0.4 $\mu\text{g/mL}$. It should be noted CLSI tables do not provide a separate breakpoint for oxytetracycline, but CLSI provides a breakpoint for tetracycline ($\leq 2 \mu\text{g/mL}$), 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 10^7 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

191 virtual eradication by 24 h (Fig. 3). In serum, the killing action at 24 h was less marked at 2x
192 and 4x MIC than in MHB, with some re-growth occurring. The in vitro killing action was
193 judged to be co-dependent for both matrices (Fig. 3). Ex vivo time-kill curves are shown in
194 Appendix B.

195

196 *Binding to serum protein*

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

200

201 **Discussion**

202 Many previous studies have shown that, for some drugs of the macrolide/triamilide
203 groups, MICs determined in serum are much higher than those determined using the broths
204 recommended in CLSI and EUCAST guidelines. An example is tulathromycin; for this drug
205 Toutain et al. (2016) reported MICs some 50 times lower in bovine serum than in broths for
206 *M. haemolytica* and *P. multocida* of bovine origin. When serum values were corrected for
207 drug binding to serum protein, differences were even greater; causes have not been positively
208 determined. In contrast, in this study the 'serum effect' was reversed; MICs were higher in
209 broth than in serum. Toutain et al. (2016) showed clearly that accuracy of MIC determination,
210 to be used in dose prediction, can be obtained from the approved broth MIC data by applying
211 a robust scaling factor to bridge in vitro MHB to in vivo relevant serum/plasma/blood values.
212 Oxytetracycline MICs were reported against a single bovine isolate of *M. haemolytica* (A1
213 76/1), in five matrices (Brentnall et al., 2012, 2013); values (µg/mL) were 0.5 (MHB), 0.8
214 (cation adjusted MHB), 14.8 (serum), 12.8 (exudate) and 11.2 (transudate). Therefore, MIC
215 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%s 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.

290

291 Despite these considerations, in immunocompetent animals with pneumonic

292 infections, even the limited direct killing activity in serum demonstrated in this study might

293 contribute to efficacy, particularly in those cases with mild infection, treated early, in which

294 biophase bacterial counts would normally be low. Epidemiological data on oxytetracycline

295 MICs have indicated a bimodal distribution (Yoshimura et al., 2001). Even allowing for these

296 MICs, measured conventionally in an artificial growth matrix, some 40-50% of isolates had

297 MICs of 8.0 or 16.0 µg/mL for calf strains of *M. haemolytica* and *P. multocida*. On the other

298 hand, 30 to 40% of isolates had broth MICs of 0.50 µg/mL; the equivalent serum MIC, from

299 the present data, would be of the order of 12.5 µg/mL, which is approximately two to three

300 times higher than maximum serum concentrations of oxytetracycline achieved in calves with

301 the recommended dose rate of 20 mg/kg (Nouws and Vree, 1983; Toutain and Raynaud,

302 1983; Nouws et al., 1990; Brentnall et al., 2013). Alternative mechanisms of action of

303 oxytetracycline are shown in Appendix C.

305 Conclusions

306 Concentrations of oxytetracycline in serum and broth were not measured at the start

307 and completion of the in vitro studies; it is possible that reported differences between the

308 media might have been due, in part, to some degradation of the drug, but at differing rates

309 over the 24 h incubation periods. Differing bacterial growth rates in the two media are

310 possible, even likely, and this could contribute to the reported differences. Time-kill studies

311 were based on fixed concentrations for a pre-defined time period. In vivo, concentrations in

312 serum and the biophase first increase and then decrease after systemic, non-vascular dosing.

313 Therefore, in vitro time-kill methods, such as hollow fibre models, better reflect the

314 circumstances of clinical exposure and could be used in future studies.

315

316 **Conflict of interest statement**

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

325

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 328 and Rural Affairs (DEFRA). Oxytetracycline was supplied by Norbrook Laboratories.

329

330 **Appendix: Supplementary Material**

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

333

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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).

445 **Table 1.** Oxytetracycline geometric mean standard deviation (SD) minimum inhibitory
 446 concentrations (MICs) and minimum bactericidal concentrations (MBCs) measured in
 447 Mueller Hinton broth (MHB) and serum, along with MIC:MBC and MHB:serum ratios ($n =$
 448 6).

Matrix	MIC ($\mu\text{g/mL}$)	MBC ($\mu\text{g/mL}$)	MBC:MIC ratio
<i>Mannheimia haemolytica</i>			
MHB	0.22 (0.12)	2.83 (2.51)	13.1:1
Serum	5.46 (4.61) ^b	10.08 (6.20) ^a	1.9:1
Serum:MHB ratio	25.2:1	3.6:1	
<i>Pasteurella multocida</i>			
MHB	0.25 (0.08)	1.26 (1.33)	5.1:1
Serum	6.75 (2.58) ^b	12.67 (4.13) ^b	1.9:1
Serum:MHB ratio	27.4:1	10.1:1	

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

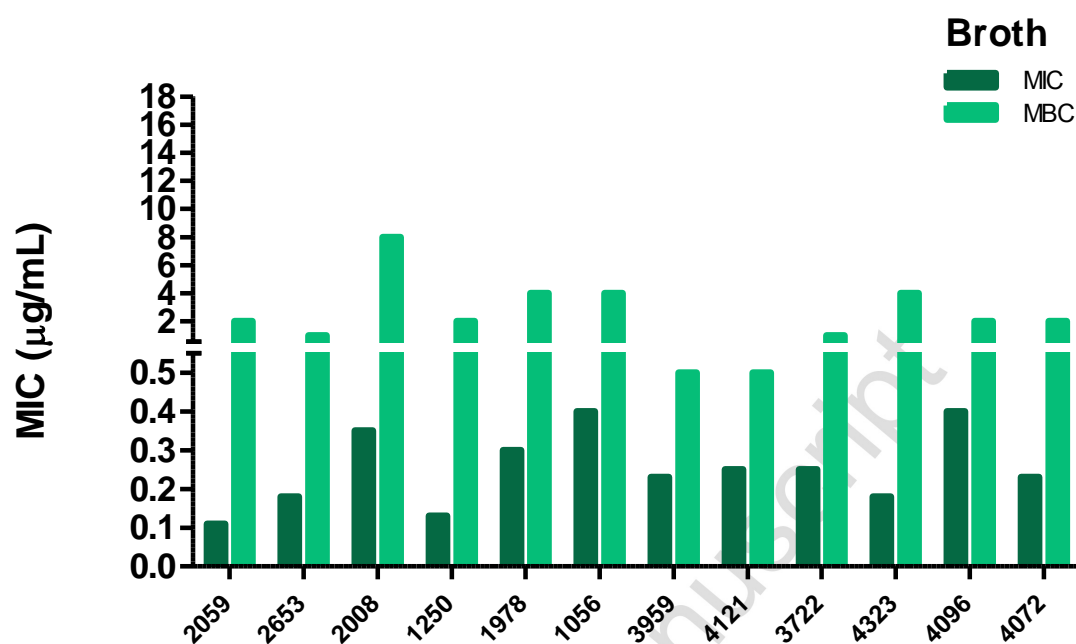
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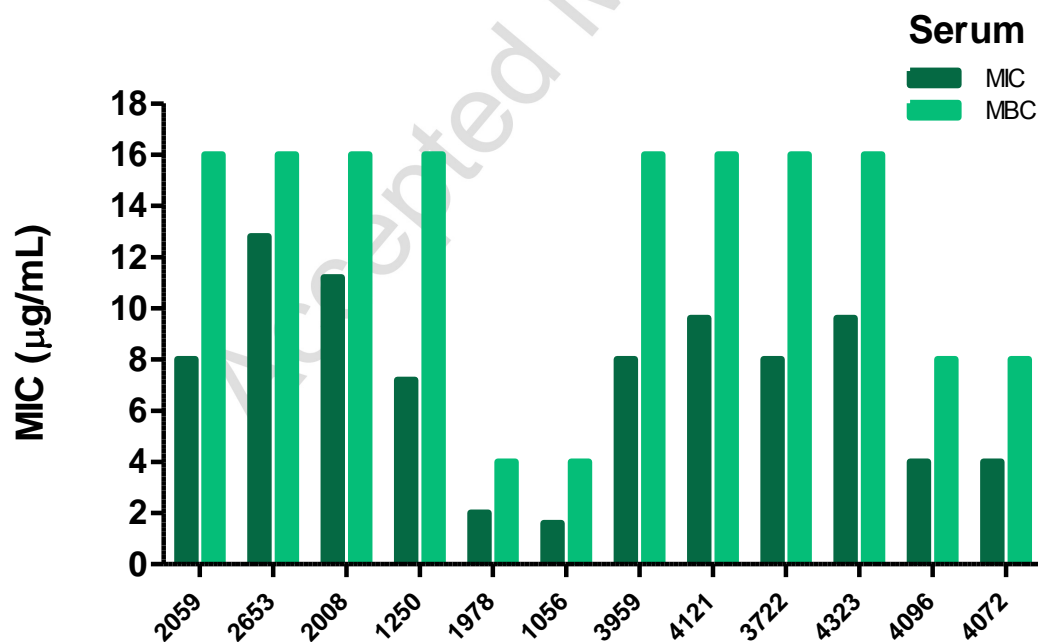
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453 Figure 1.

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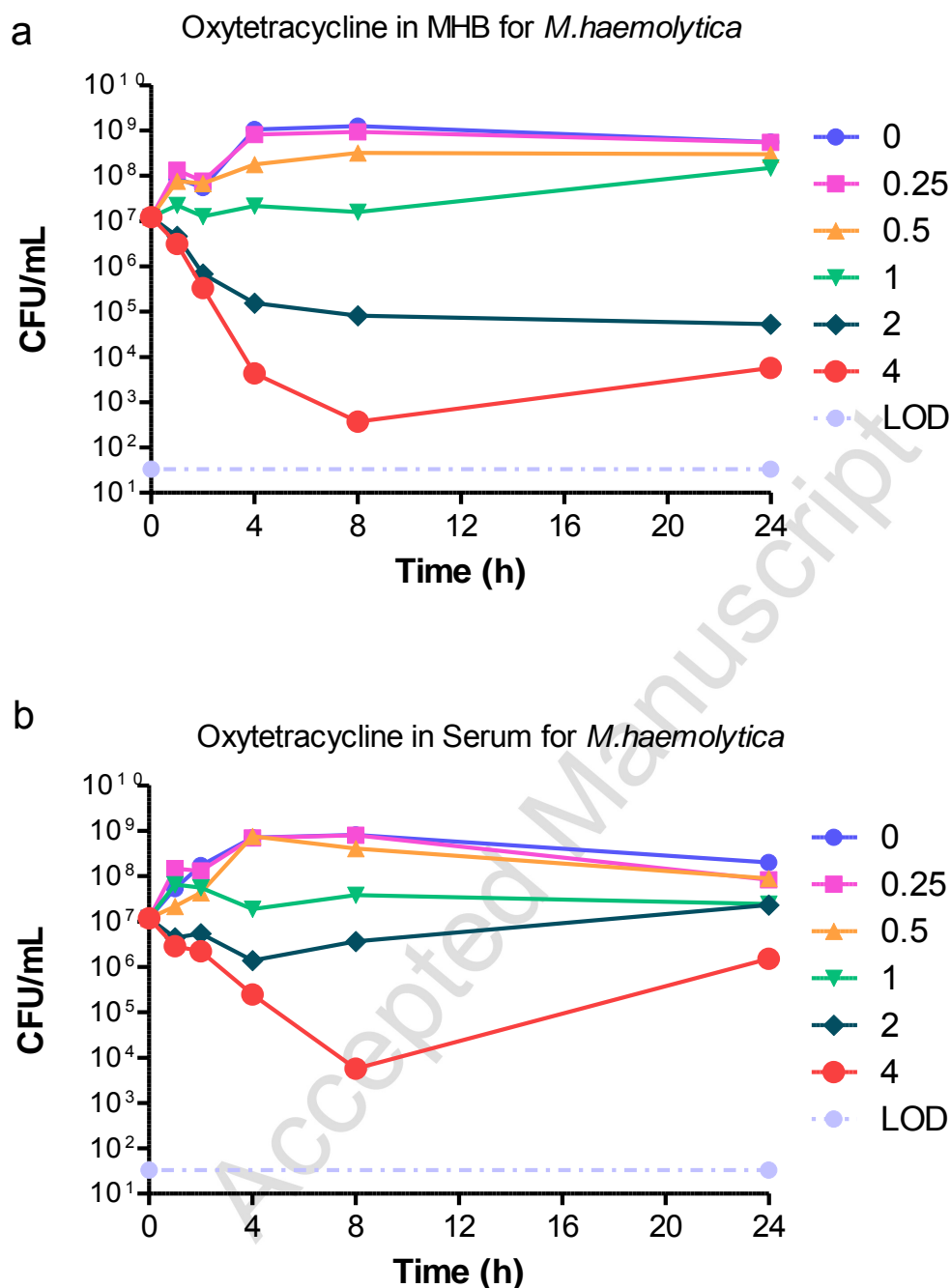


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459 Figure 2.



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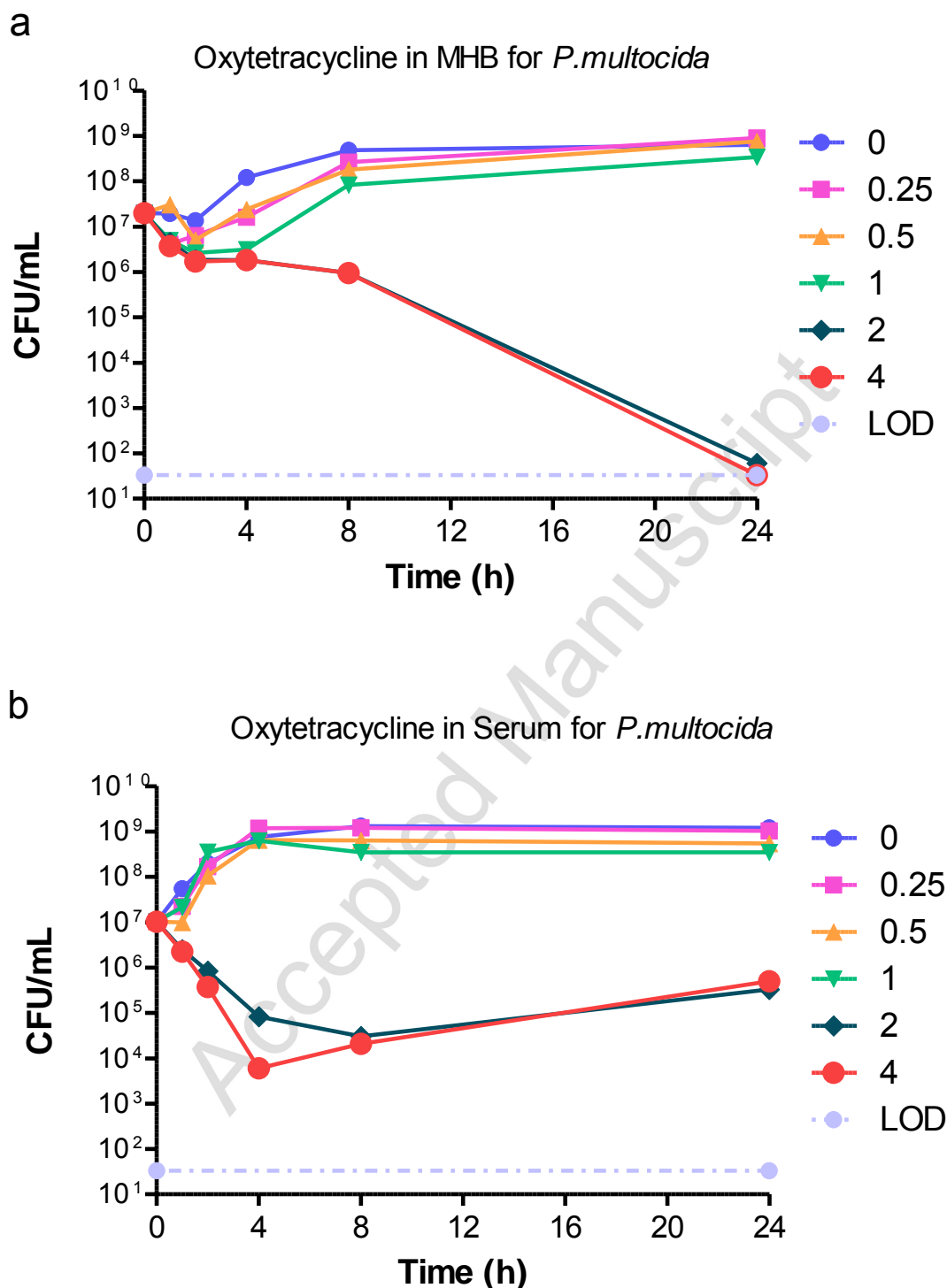
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466 Figure 3.



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