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Title: 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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1 **Comparison of standardised versus non-standardised methods for testing the in vitro**
2 **potency of oxytetracycline against *Mannheimia haemolytica* and *Pasteurella multocida***
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4
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17 **Highlights**

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

26 **Abstract**

27 The in vitro pharmacodynamics of oxytetracycline were established for six isolates of
28 each of the calf pneumonia pathogens *Mannheimia haemolytica* and *Pasteurella multocida*.
29 Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and
30 bacterial time-kill curves were determined in two matrices, Mueller Hinton broth (MHB) and
31 calf serum. Geometric mean MIC ratios, serum:MHB, were 25.2:1 (*M. haemolytica*) and
32 27.4:1 (*P. multocida*). The degree of binding of oxytetracycline to serum protein was 52.4%.
33 Differences between serum and broth MICs could not be accounted for by oxytetracycline
34 binding to serum protein. In vitro time-kill data suggested a co-dependent killing action of
35 oxytetracycline. The in vitro data indicate inhibition of the killing action of oxytetracycline by
36 serum factor(s). The nature of the inhibition requires further study. The outcome of treatment
37 with oxytetracycline of respiratory tract infections in calves caused by *M. haemolytica* and *P.*
38 *multocida* may not be related solely to a direct killing action.

39

40 *Keywords: Mannheimia haemolytica; Pasteurella multocida*; Bovine; Oxytetracycline;
41 Pharmacodynamics

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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

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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

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92 of serum on MHB MICs by combining the two matrices in varying proportions; (3) to
93 determine the effect of low, intermediate and high bacterial counts on oxytetracycline MICs;
94 (4) to compare in vitro time-kill curves for oxytetracycline in MHB and calf serum; and (5) to
95 determine the degree of binding of oxytetracycline to protein in calf serum.
96

97 **Materials and methods**

98 *Origin, storage, selection and culture of bacterial isolates*

99 Twenty isolates of each of two calf pathogens, *M. haemolytica* and *P. multocida*, were
100 obtained post-mortem from field cases of calf pneumonia in various geographical regions of
101 the United Kingdom. They were supplied on swabs by the Veterinary Laboratories Agency
102 (AHVLA), now Animal and Plant Health Agency (APHA), and stored at -70 °C in
103 glycerol:milk:water (20:10:70). This fluid was boiled for 5 s, left to cool for 12 h and then
104 boiled again for a further 5 s.
105

106 Two criteria were used to select 6/20 isolates of each of the two bacterial species for
107 further study: (1) each isolate was investigated for its ability to grow logarithmically in four
108 fluids (MHB and calf serum, exudate and transudate); (2) each isolate was evaluated for
109 susceptibility to oxytetracycline by disc diffusion and measurement of diameter of zone of
110 growth inhibition. Since tetracycline but not oxytetracycline is listed in the CLSI standards,
111 this measurement was not determined according to CLSI (2008). MICs were then determined
112 in MHB, using doubling dilutions.
113

114 Culture methods and bacterial viability counts, determined by serial dilution and spot-
115 plate counts, were as described by Lees et al. (2015).
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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).
130
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.
139
140 *Antimicrobial growth (time-kill) curves*

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141 For six isolates each of *M. haemolytica* and *P. multocida,* in vitro time-kill curves
142 were established using oxytetracycline concentrations corresponding to 0.25, 0.5, 1, 2 and 4x
143 multiples of MIC in both MHB and calf serum, as previously described (Lees et al., 2015).
144 The lowest quantifiable count was 33 colony forming units (CFU)/mL. Ex vivo analyses were
145 performed as described in Appendix B.
146
147 *Serum protein binding of oxytetracycline*
148 The degree of binding of oxytetracycline to serum protein in vivo was determined in
149 triplicate on pooled samples from 10 calves harvested from a tissue cage study for seven
150 concentrations, ranging from 0.43 to 2.07 μg/mL. The total concentration was measured on
151 each sample as described in Appendix B and binding to protein was determined on a second
152 aliquot of each sample by ultracentrifugation at 4,000 *g* and 25 °C for 20 min. The
153 ultrafiltration device used was an Amicon Ultra Centrifugal filter (Ultracel 10 K, Millipore)
154 and oxytetracycline concentrations were re-determined on the ultrafiltrate.
155
156 *Statistical analyses*
157 MIC and MBC data are presented as geometric means and standard deviation (SD).
158 Differences in MIC and MBC values between MHB and serum were compared with a paired *t*
159 test or the non-parametric Wilcoxon test, depending on whether the data passed a normality
160 test.
161
162 **Results**
163 *Selection of isolates*
164 Six isolates of each species were selected to satisfy two criteria. Firstly, the
165 percentages growing logarithmically were 65, 65, 40 and 55 for *M. haemolytica* and 90, 75,

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166 65 and 65 for *P. multocida* for MHB, calf serum, exudate and transudate, respectively.
167 Secondly, initial MIC studies using doubling dilutions indicated that the MIC for MHB was ≤
168 0.4 µg/mL. It should be noted CLSI tables do not provide a separate breakpoint for
169 oxytetracycline, but CLSI provides a breakpoint for tetracycline (≤ 2 µg/mL), and indicates
170 that the breakpoint interpretation for tetracycline also applies to oxytetracycline. Therefore,
171 the oxytetracycline MICs were less than the tetracycline breakpoint. The six isolates of each
172 species selected comprised highest, lowest and four with intermediate MICs.
173
174 *Minimum inhibitory and minimum bactericidal concentrations*
175 MICs of the 12 selected isolates were re-determined separately in MHB and serum.
176 MICs and MBCs are illustrated in Fig. 1. Table 1 presents geometric mean MICs and MBCs,
177 and ratios MBC:MIC. The potency of oxytetracycline, expressed as MIC, was 25.2 times
178 greater in MHB for *M. haemolytica* and 27.4 times greater in MHB for *P. multocida*,
179 compared to serum MICs. Therefore, potency differed markedly between the two growth
180 matrices. Using MBC as the indicator of potency indicated smaller differences than for MIC,
181 but again in favour of MHB.
182
183 *Time-kill curves*
184 Starting inoculum counts of the order of 10^7 CFU/mL were selected to reflect a
185 moderate to high bacterial load in clinical subjects (Roof, 2011). Despite marked differences
186 in MICs between MHB and serum, growth inhibition curves in these matrices using multiples
187 of MIC were broadly similar for *M. haemolytica* (Fig. 2). However, reductions in count were
188 smaller in serum than in MHB at MIC multiples of 2.0 and 4.0. With both matrices, some re-
189 growth occurred at 24 h. The in vitro killing pattern was classified as co-dependent (on both
190 concentration and time). For *P. multocida* in MHB, MIC multiples of 2.0 and 4.0 produced

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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

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216 the other, whilst similar values were obtained for the two artificial matrices and similar values
217 were obtained for the three biological fluids.
218
219 These findings were confirmed and extended to demonstrate: (1) much higher MICs of
220 oxytetracycline in serum compared to MHB for *M. haemolytica* and *P. multocida*; (2) higher
221 oxytetracycline MBCs in serum compared to MHB, but with lower serum:MHB ratios for
222 MBC than for MIC. This study also quantified inter- and intra-species differences in
223 variability for six isolates of each species; coefficient of variation (CV%) serum values for *M.*
224 *haemolytica* were 84 (MIC) and 62 (MBC). Corresponding CV%s for *P. multocida* were
225 lower (38 MIC; 33 MBC). Considering the serum MIC values reported in this study, and
226 species and isolate variability, it is important for future studies to recognise that we used
227 bovine serum from a single source. Breed, age, sex, disease state, country and other factors
228 might provide MIC differences between sera, even from a single species. Such differences
229 should be quantified. However, this paper had a more limited immediate goal, to compare
230 broth with serum from the target species derived from one source only.
231
232 The cause(s) of serum/MHB differences in MIC for oxytetracycline have not been
233 established. In a recent study, albumin concentrations in MHB and calf serum were 0.033 and
234 32.2 g/L, thus differing by approximately 1,000-fold (Brentnall et al., 2012). Most serum
235 protein binding occurs to albumin and it is very likely that total and free concentrations of
236 oxytetracycline in MHB were identical. Therefore, approximately two-fold higher MIC values
237 in serum compared to MHB would be anticipated from the binding of oxytetracycline to
238 serum protein, which was shown to be 52.4% of total concentration. This confirms the 50%
239 binding described by Pilloud (1973) and is intermediate between 18.6% (Ziv and Sulman,
240 1972) and 72% (Nouws et al., 1985) described for cattle by other authors. Cause(s) of

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241 differing degrees of protein binding in these studies are not known, but it should be noted that
242 the degree of binding has an impact on dosages required to achieve a given level of efficacy.
243 The prediction of approximately two-fold higher MICs in serum in the present study arises
244 because protein bound AMDs are microbiologically inactive (Wise, 1986; Zeitlinger et al.,
245 2004). This relatively small (two-fold) predicted difference is well short of the 25- to 27-fold
246 experimentally determined differences in MIC between MHB and serum for *M. haemolytica*
247 and *P. multocida,* respectively.
248

249 In quantitative terms, for *P. multocida*, correction for protein binding yields a mean
250 fraction unbound (fu) serum MIC of 3.21 μg/mL, whilst broth MIC was 0.25 μg/mL; thus, the
251 mean fu serum MIC is 12.9-fold greater than the broth MIC, which is the CLSI and EUCAST
252 and therefore universally accepted standard. For *M. haemolytica*, the fu serum MIC was 11.8
253 times greater than broth MIC. The data indicate inhibition of the killing action of
254 oxytetracycline by some serum factor(s). The data demonstrate antagonism of the action of
255 oxytetracycline beyond what can be ascribed to non-specific protein binding.
256

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

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265 the marked differences in MIC between MHB and cation adjusted MHB on the one hand and
266 calf serum on the other require alternative explanations and further study.
267
268 Honeyman et al. (2015) compared MICs of several tetracyclines in broth and 50%
269 broth:50% mouse serum as matrices. For a strain of *Streptococcus pneumoniae,* MICs were
270 identical for six compounds but, with added serum, 2-4 fold increases were obtained for five,
271 whilst MIC was increased 32-fold for one compound. In contrast, for a strain of
272 *Staphylococcus aureus,* MIC was increased in the broth:serum combined matrix relative to
273 broth for all 12 compounds investigated and, for seven, the increase was in the range 8- to
274 128-fold.
275
276 The PDs of oxytetracycline was further investigated in time-kill studies. Using
277 multiples of up to 4x MIC indicated a probable co-dependent killing mechanism that is
278 dependency on both concentration and time. However, confirming the type of killing action
279 would benefit from further studies using higher multiples of MIC than the five used in this
280 study.
281
282 These data suggest that serum, exudate and transudate may be useful alternatives to
283 broth for potency determination, when the objective is estimation of a dose for clinical use,
284 based on PK/PD modelling approaches. These biological fluids are not identical to pulmonary
285 epithelial lining fluid, but are much closer in composition to the latter than artificial broths.
286 Further refinement of the methodology used in this study would be to determine potency in
287 serum in the presence of other ‘natural’ constituents, such as leucocytes and antibodies, as
288 well as the normal bacterial flora that compete with pathogens. Ideally, although technically
289 difficult, it would also be relevant to determine potency in pulmonary epithelial ling fluid.

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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.

304

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.

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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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423 **Figure legends**

424

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

429

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

435

436 Fig 3. In vitro inhibition of growth of *Pasteurella multocida* over 24 h exposure to five
437 minimum inhibitory concentrations (MIC) multiples (0.25 to 4.0) of oxytetracycline,
438 measured in either Mueller Hinton broth (MHB) or serum: (a) MHB and (b) serum (mean for
439 six isolates in each matrix). Standard error of the mean (SEM) bars not included for clarity.
440 Dotted line indicates limit of quantification (33 colony forming units, CFU/mL).

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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 (µg/mL)	MBC (µg/mL)	MBC:MIC ratio
Mannheimia haemolytica			
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	
Pasteurella multocida			
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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453 Figure 1.

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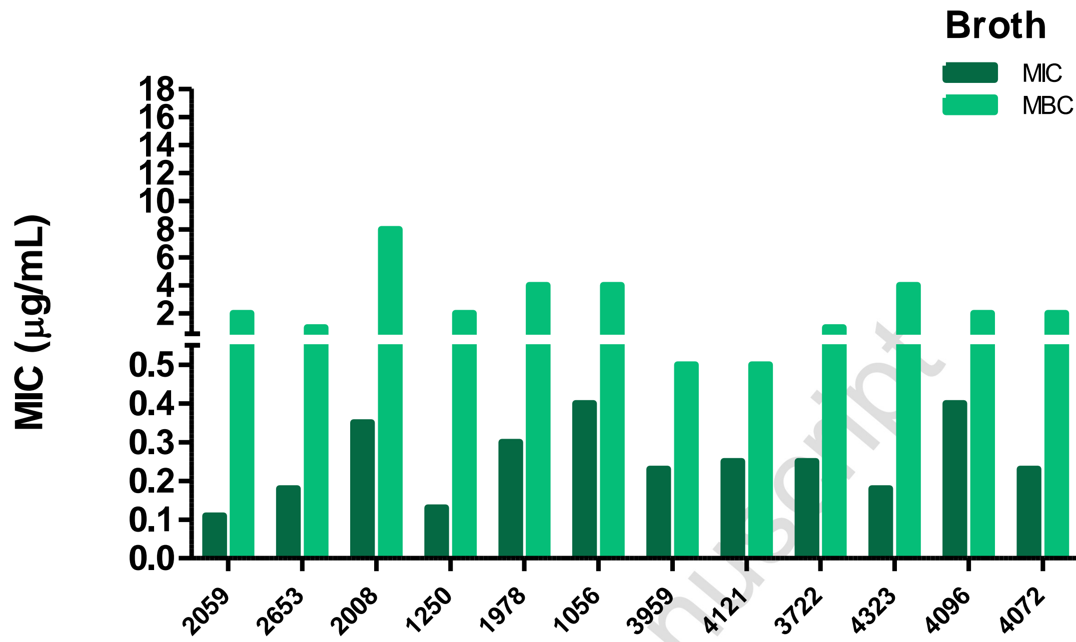

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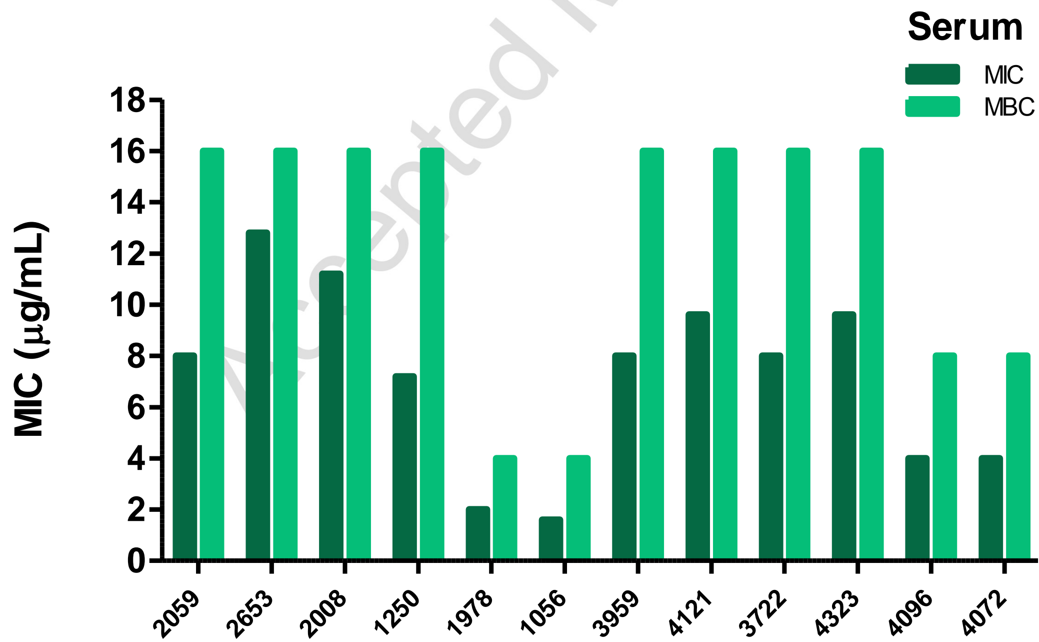

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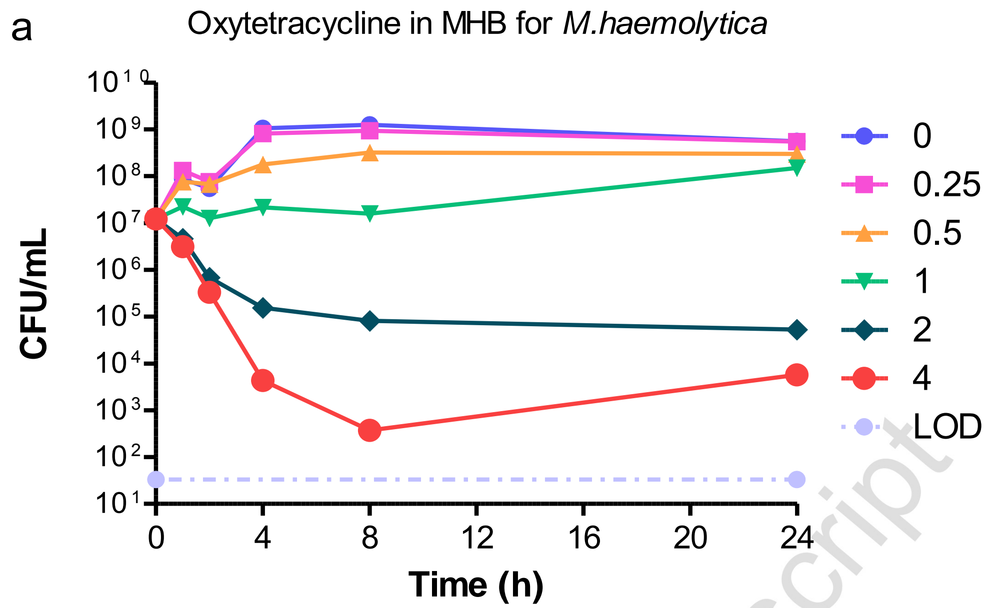
459 Figure 2.



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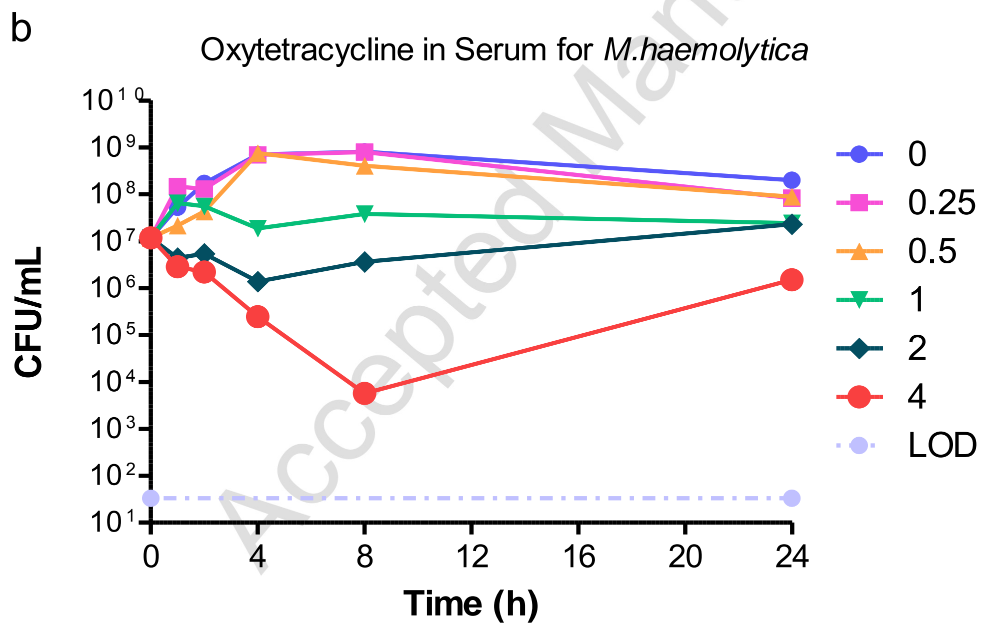

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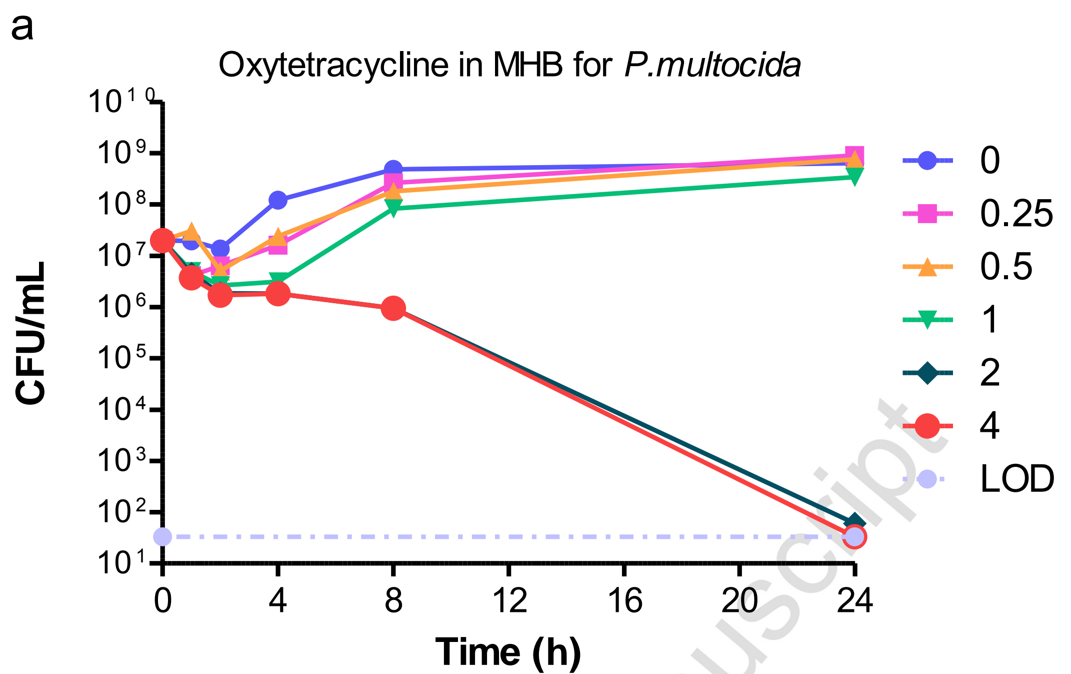
466 Figure 3.



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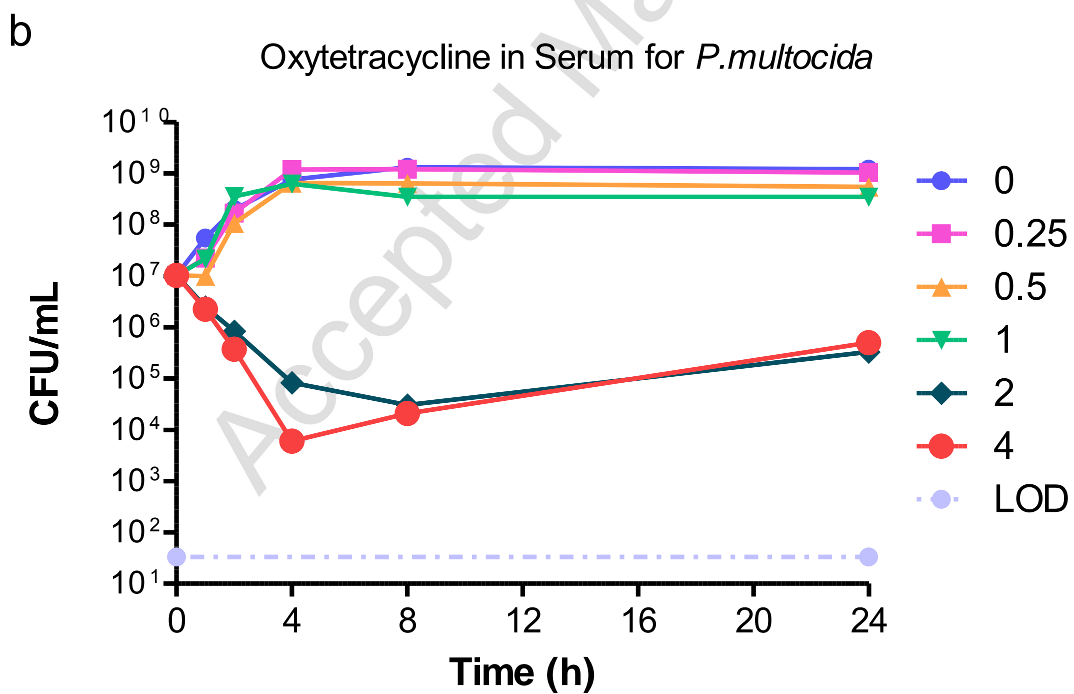

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