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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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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.

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

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

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).

116

Minimum inhibitory and minimum bactericidal concentrations

117

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*

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,

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

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

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

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

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.

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.

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.

315

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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329

Appendix: Supplementary Material

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

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333

References

335   Aliabadi, F.S., Lees, P., 2001. Pharmacokinetics and pharmacodynamics of danofloxacin in
336   serum and tissue fluids of goats following intravenous and intramuscular
337   administration. American Journal of Veterinary Research 62, 1979-1989.
338
339   Aliabadi, F.S., Lees, P., 2002. Pharmacokinetics and pharmacokinetic/pharmacodynamic
340   integration of marbofloxacin in calf serum, exudate and transudate. Journal of
341   Veterinary Pharmacology and Therapeutics 25, 161-174.
342

343 Brentnall, C., Cheng, Z., McKellar, Q.A., Lees, P., 2012. Pharmacodynamics of
344   oxytetracycline administered alone and in combination with carprofen in calves.
345   Veterinary Record 171, 273.
346
347 Brentnall, C., Cheng, Z., McKellar, Q.A., Lees, P., 2013. Pharmacokinetic-pharmacodynamic
348   integration and modelling of oxytetracycline administered alone and in combination
349   with carprofen in calves. Research in Veterinary Science 94, 687-694.
350
351 CLSI, 2008. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests
352   for Bacteria Isolated from Animals: Approved Standard - 3rd Edn. CLSI document
353   M31-A3.
354
355 Davey, L.A., Ferber, M.T., Kaye, B., 1985. Comparison of the serum pharmacokinetics of a
356   long acting and a conventional oxytetracycline injection. Veterinary Record 117, 426-
357   429.
358
359 Esaki, H., Asai, T., Kojima, A., Ishihara, K., Morioka, A., Tamura, Y., Takahashi, T., 2005.
360   Antimicrobial susceptibility of *Mannheimia haemolytica* isolates from cattle in Japan
361   from 2001 to 2002. Journal of Veterinary Medical Sciences 67, 75-77.
362
363 Honeyman, L., Ismail, M., Nelson, M.L., Bhatia, B., Bowser, T.E., Chen, J., Mechiche, R.,
364   Ohemeng, K., Verma, A.K., Cannon, E.P., Macone, A., Tanaka, S.K., Levy, S., 2015.
365   Structure-activity relationship of the aminomethylcyclines and the discovery of
366   omadacycline. Antimicrobial Agents and Chemotherapy 59, 7044-7053.
367
368 Lees, P., Pelligand, L., Illambas, J., Potter, T., Lacroix, M., Rycroft, A., Toutain, P.L., 2015.
369   Pharmacokinetic/pharmacodynamic integration and modelling of amoxicillin for the
370   calf pathogens *Mannheimia haemolytica* and *Pasteurella multocida*. Journal of
371   Veterinary Pharmacology and Therapeutics 38, 457-470.
372
373 Luthman, J., Jacobsson, S.O., 1983. The availability of tetracyclines in calves. Nordisk
374   Veterinaermedicin 35, 292-299.
375
376 Nouws, J.F., Breukink, H.J., Binkhorst, G.J., Lohuis, J., van Lith, P., Mevius, D.J., Vree,
377   T.B., 1985. Comparative pharmacokinetics and bioavailability of eight parenteral
378   oxytetracycline-10% formulations in dairy cows. Veterinary Quarterly 7, 306-314.
379
380 Nouws, J.F., Smulders, A., Rappalini, M., 1990. A comparative study on irritation and residue
381   aspects of five oxytetracycline formulations administered intramuscularly to calves,
382   pigs and sheep. Veterinary Quarterly 12, 129-138.
383
384 Nouws, J.F., Vree, T.B., 1983. Effect of injection site on the bioavailability of an
385   oxytetracycline formulation in ruminant calves. Veterinary Quarterly 5, 165-170.
386
387 Pilloud, M., 1973. Pharmacokinetics, plasma protein binding and dosage of oxytetracycline in
388   cattle and horses. Research in Veterinary Science 15, 224-230.
389
390 Roof, C., 2011. Qualification and quantification of bacterial pathogen load in acute bovine
391   respiratory disease cases. Masters Thesis, Kansas State University, Manhattan,
392   Kansas, USA.

393

394 Sidhu, P., Shojaee Aliabadi, F., Andrews, M., Lees, P., 2003. Tissue chamber model of acute
395 inflammation in farm animal species. Research in Veterinary Science 74, 67-77.

396

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

400

401 Toutain, P.L., Raynaud, J.P., 1983. Pharmacokinetics of oxytetracycline in young cattle:
402 Comparison of conventional vs long-acting formulations. American Journal of
403 Veterinary Research 44, 1203-1209.

404

405 Toutain, P.-L., Potter, T., Pelligand, L., Lacroix, M., Illambas, J., Lees, P., 2016. Standard
406 PK/PD concepts can be applied to determine a dosage regimen for a macrolide: The
407 case of tulathromycin in the calf. Journal of Veterinary Pharmacology and
408 Therapeutics doi: 10.1111/jvp.12333.

409

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

412

413 Yoshimura, H., Ishimaru, M., Endoh, Y.S., Kojima, A., 2001. Antimicrobial susceptibility of
414 *Pasteurella multocida* isolated from cattle and pigs. Journal of Veterinary Medicine B
415 Infectious Disease Veterinary Public Health 48, 555-560.

416

417 Zeitlinger, M.A., Sauermann, R., Traunmuller, F., Georgopoulos, A., Muller, M., Joukhadar,
418 C., 2004. Impact of plasma protein binding on antimicrobial activity using time-killing
419 curves. Journal of Antimicrobial Chemotherapy 54, 876-880.

420

421 Ziv, G., Sulman, F.G., 1972. Binding of antibiotics to bovine and ovine serum. Antimicrobial
422 Agents and Chemotherapy 2, 206-213.

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).

441
442
443
444

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	

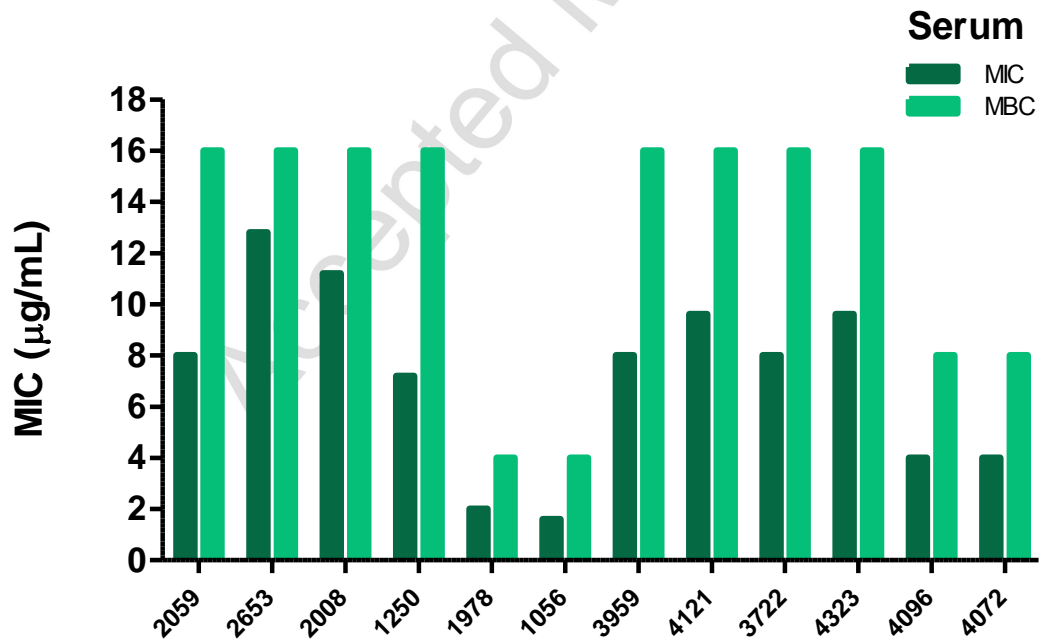449 Significant difference between MHB and serum: [a] $P < 0.05$; [b] $P < 0.01$.

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452

453   Figure 1.

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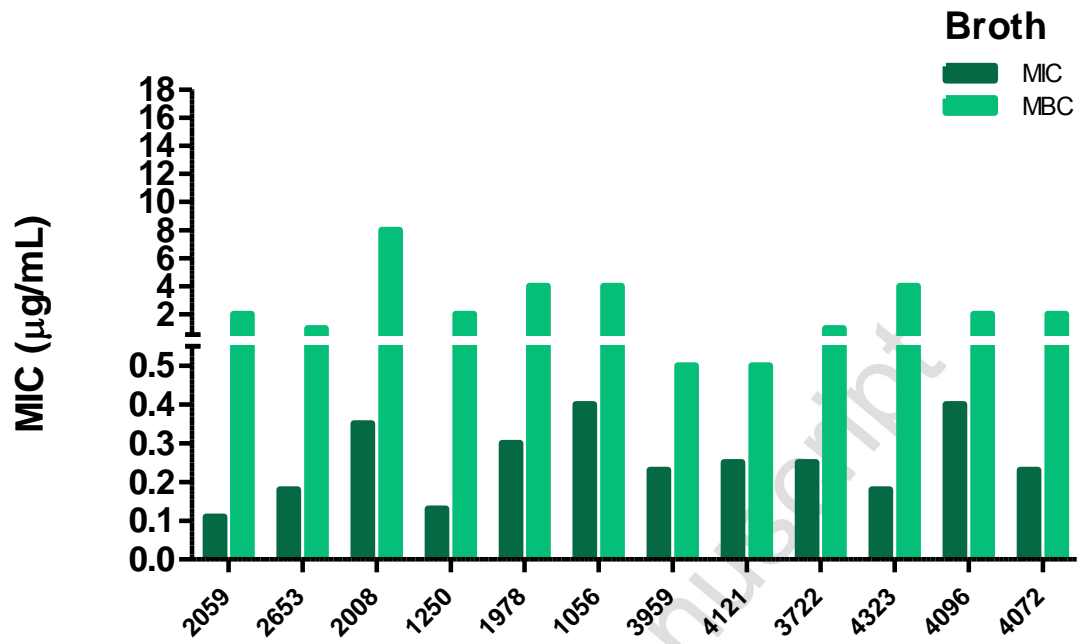
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459   Figure 2.

a

Oxytetracycline in MHB for *M.haemolytica*

460

b

Oxytetracycline in Serum for *M.haemolytica*
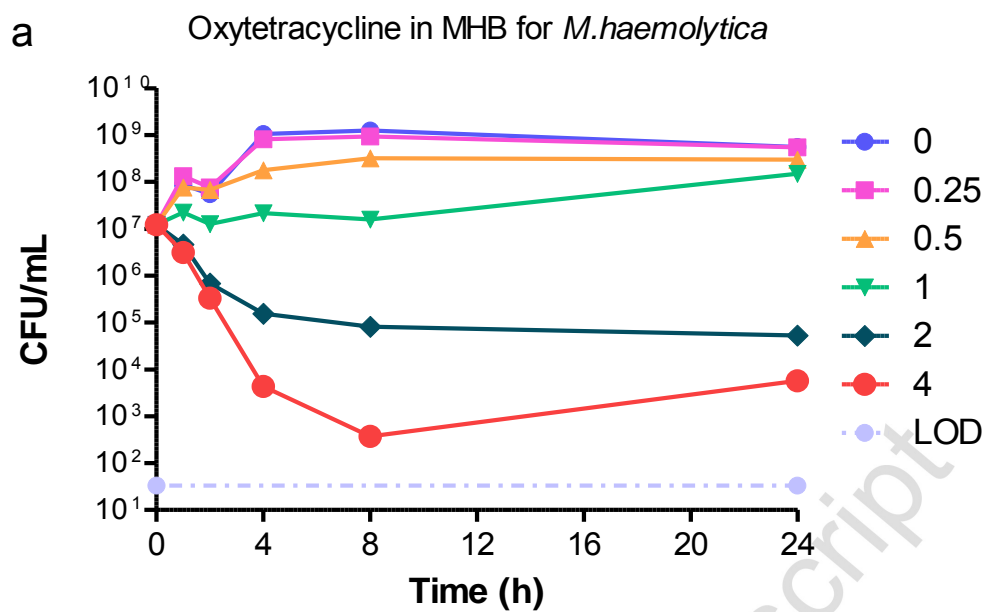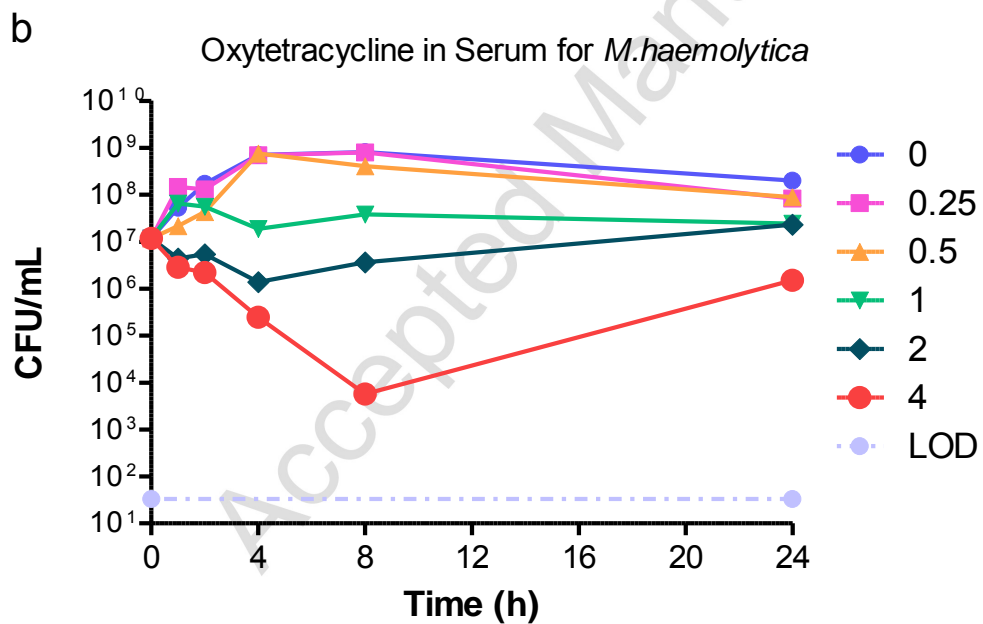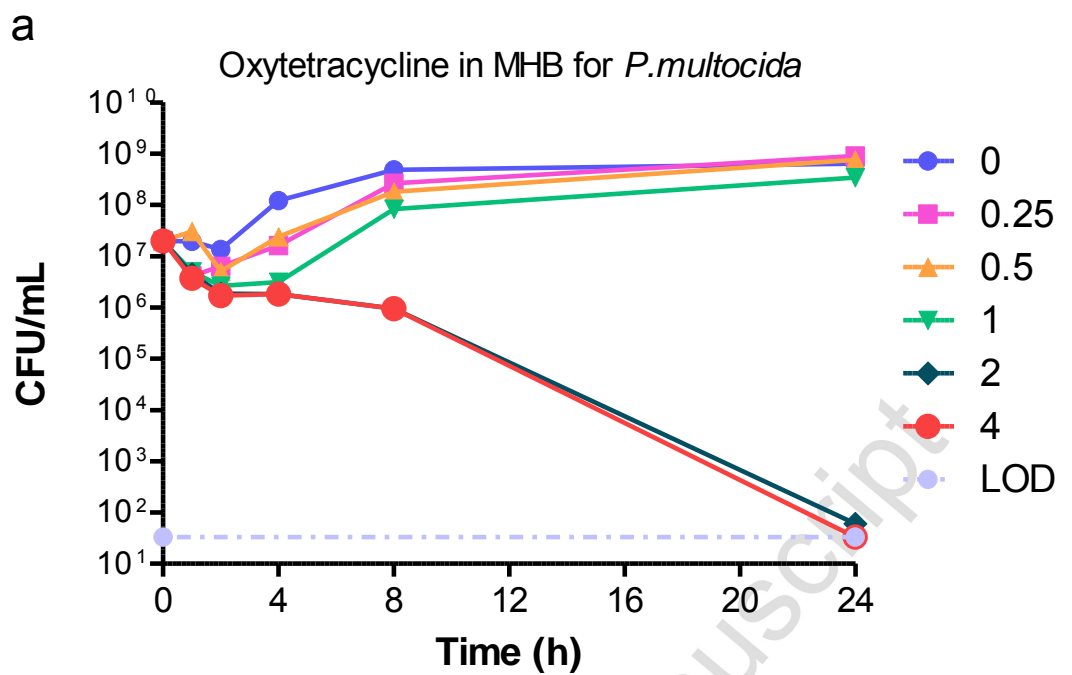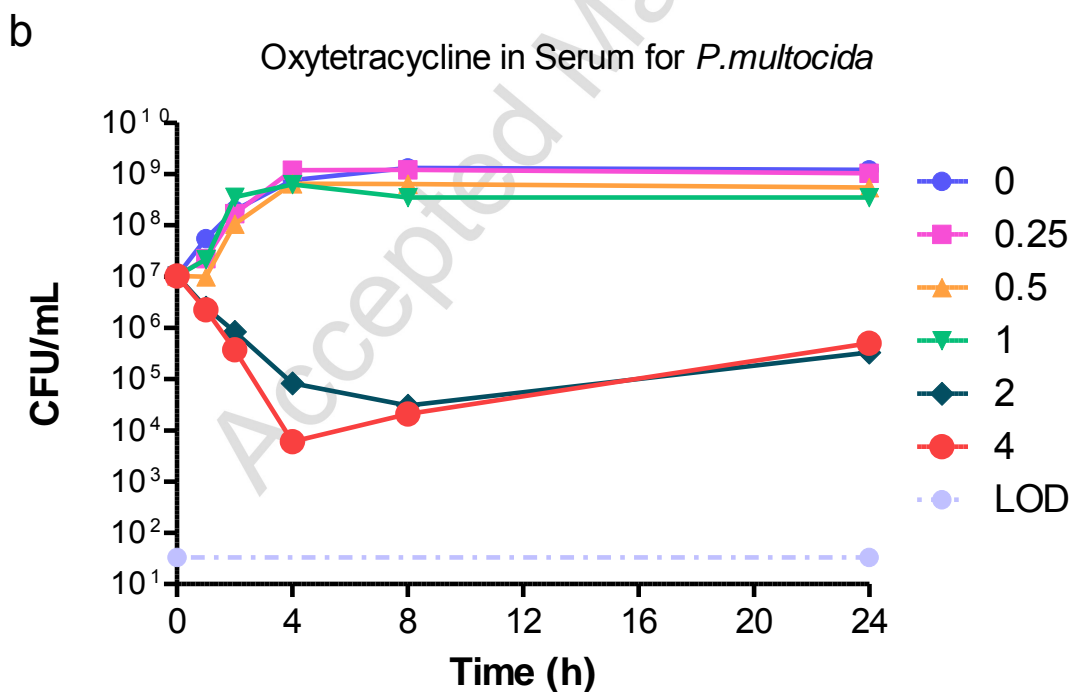
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462

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464

465

466   Figure 3.

a

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b

468

469

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