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Original Article

Activity of florfenicol for the porcine pneumonia pathogens *Actinobacillus pleuropneumoniae* and *Pasteurella multocida* using standardised versus non-standardised methodology

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Abstract

Four indices of antimicrobial potency were determined for florfenicol and the pig pneumonia pathogens, *Actinobacillus pleuropneumoniae* and *Pasteurella multocida*. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), mutant prevention concentration (MPC) and time-kill curves were determined in two matrices, broth and pig serum. Five overlapping sets of two-fold dilutions were used to increase accuracy of the measurements. MIC and MBC serum:broth ratios for *A. pleuropneumoniae* were 0.96:1 and 1.07:1, respectively, and corresponding values for *P. multocida* were 0.72:1 and 0.50:1. The percentage binding of florfenicol to serum protein was 65.4%, and fraction unbound (fu) serum MICs were significantly lower, 2.71-fold and 3.82-fold, respectively, than predicted for free serum concentrations for *A. pleuropneumoniae* and *P. multocida*. Similar culture medium differences were obtained for MBC and MPC. MICs in serum and broth were increased significantly and progressively for high, medium and low initial inoculum counts. Serum MPC:MIC ratios for *A. pleuropneumoniae* and *P. multocida* were 12.5:1 and 13.6:1, respectively; ratios for broth were similar. The killing action of florfenicol had the characteristics of concentration dependency for both species in both growth media. These data indicate the value of using a biological medium, when determining microbiological potency indices, to predict dosage for clinical use.

Keywords

Minimum inhibitory concentration; Mutant prevention concentration; Florfenicol; *Actinobacillus pleuropneumoniae*; *Pasteurella multocida*.

Introduction

Florfenicol has been used in pigs for the therapy of pneumonia caused by the microorganisms *Actinobacillus pleuropneumoniae*, *Pasteurella multocida* and *Bordetella haemolytica* (Kehrenberg et al., 2008). In vitro studies have demonstrated bactericidal, concentration-dependent activity at concentrations close to the minimum inhibitory concentration (MIC) against some veterinary pathogens (Illambas et al., 2013; Sidhu et al., 2014).

Commonly used indices defining the potency of antimicrobial drugs are MIC, the lowest In vitro concentration inhibiting visible growth, and minimum bactericidal concentration (MBC), the lowest concentration producing a 3log₁₀ reduction in bacterial count. Time-kill curves, quantifying the time course of growth inhibition, and as a separate measure mutant prevention concentration (MPC), are also used. The latter is the lowest drug concentration preventing the growth of the least susceptible cells in high-density bacterial populations (Blondeau et al., 2004).

The standardised methodologies for MIC determination are described in EUCAST (European Committee on Antimicrobial Susceptibility Testing) and CLSI (Clinical and Laboratory Standards Institute) guidelines [VET01-A3 (formerly M31-A3)]. These ensure standardisation and reproducibility between individual workers, laboratories and across both geographical regions and time (Papich, 2013). However, for the objectives of the present study, these guidelines and standards have limitations. Regarding accuracy, MICs are determined using two-fold dilutions, giving a potential for up to 100% error on single isolate estimates. For many purposes, this is wholly acceptable, and indeed is necessary, when large numbers of

isolates are examined to establish MIC distributions. When plotted on a histogram, using a log-base 2 distribution, the distributions are log-normal. These histograms facilitate the identification of wild-type distributions. The CLSI and EUCAST standards thereby enable determination of ECOFF and epidemiological breakpoints.

Nevertheless, the lack of accuracy is a concern when MICs are to be correlated with pharmacokinetic data, as a basis for dose determination. Therefore, based on methods previously described (Aliabadi and Lees, 2001; Sidhu et al., 2010) five overlapping sets of two-fold dilutions were used in this study to ensure accuracy within 20% of the true value for the small number of isolates studied, compared to the much larger numbers use to establish distributions.

International guidelines and standards necessarily require the use of artificial broths, e.g. Cation Adjusted Mueller Hinton Broth (CAMHB) for MIC determinations. Whilst each broth is formulated to optimize the growth of bacteria of a given species, they differ in composition, chemically and immunologically, to fluids encountered by pathogenic organisms in vivo. Hence, the quantitative determination of microbiological indices with improved accuracy and in biological fluid matrices, such as serum/plasma, can be regarded, for some drug classes and some microorganisms, as appropriate when applying pharmacokinetic/pharmacodynamic (PK/PD) integration and modelling methods to dose determination (Aliabadi et al., 2002; Brentnall et al., 2013a; Lees et al., 2015). [The terms MIC, MBC and MPC are therefore retained in this article to describe measurements in pig serum and broth.](#)

The aims of this investigation were (1) to determine the non-protein bound fraction of florfenicol in pig serum; (2) for florfenicol against six pig isolates each of *A. pleuropneumoniae* and *P. multocida* (a) to determine MICs using five sets of overlapping two-fold dilutions, (b) to compare MICs, MBCs and MPCs for florfenicol in two matrices, CAMHB and pig serum, (c) to compare MICs using low, intermediate and high starting inoculum counts, (d) to establish time-kill curves for eight multiples of MIC in broth and serum; (3) for two isolates each of *P. multocida* and *A. pleuropneumoniae* to determine the effect on florfenicol MIC of (a) varying starting inoculum pH over the range 7.0 to 8.0 (b) varying broth concentrations of calcium and magnesium and (c) addition of serum to broth in varying proportions. CLSI and EUCAST do not set standards for MBC and MPC measurements. Therefore, methods used to determine MBC and MPC in both broth and serum and MIC in serum were non-standardised.

Materials and methods

Florfenicol serum protein binding

A high pressure liquid chromatography (HPLC) method was used to analyse serum samples for florfenicol (de Craene et al., 1997). The system comprised a Beckman Ultrasphere ODS 5 μ m 4.6 x 250 mm C18 column (Beckman Coulter), Dionex Ultimate 3000 pump and autosampler connected to a Dionex UVD340S detector (Thermo Fisher). UV detection was set to wavelength 224 nm for monitoring the signal, and at 200 and 400 nm for spectral information. Chromatographic data were analysed using Chromeleon Version 6.80 and concentrations of florfenicol were calculated using ratios of peak area, compared to internal standard. The standard curve concentrations were 0, 0.0025, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5

and 10 µg/mL. Standards in serum were run between samples with every assay to confirm linearity and reproducibility.

Two sample sets were used: (1) spiked florfenicol standards in serum to determine the total concentration of protein bound and free drug; (2) an aliquot filtered using ultra-filtration devices (Amicon Ultra Centrifugal filters, Ultracel 10 k, Sigma-Aldrich) to determine the concentration in the protein-free fraction. Each sample was analysed in triplicate. HPLC analysis and ultracentrifugation were conducted at ambient temperature.

Selection of bacterial isolates

Don Whitley Scientific supplied 20 isolates of *P. multocida* and three American Type Culture Collection (ATCC) reference strains to validate MIC tests; *A. pleuropneumoniae* ATCC 27090, *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* ATCC 25922. These quality control organisms were incorporated in every MIC test; if the results did not match the ranges stated in CLSI guidelines, the experiment was repeated. Eight isolates of *A. pleuropneumoniae* were supplied by A. Rycroft (Royal Veterinary College). All *P. multocida* and *A. pleuropneumoniae* isolates were obtained from European Union (EU) field cases of pig pneumonia. They were stored at -80 °C in 10% Marvel® milk powder, 15% glycerol and sterile distilled water to 100%. The mixture was sterilized by boiling for 5 s, left to cool for 12 h and then boiled again for a further 5 s.

Six isolates of each species were selected, based on three criteria: (1) logarithmic growth in artificial broth and pig serum; (2) sensitivity to florfenicol in disk diffusion assays

(data not shown); and (3) the highest and lowest broth MICs and four isolates with intermediate MICs determined using two-fold dilutions (data not shown). This pre-selection procedure ensured that all isolates could be used in subsequent studies and that they comprised a small but diverse range of susceptible isolates.

Culture methods and bacterial counts

For *A. pleuropneumoniae* Chocolate Mueller Hinton Agar (CMHA) was used to grow the organism on a solid medium and Columbia broth supplemented with 2 µg/mL nicotinamide adenine dinucleotide (NAD) was used as the liquid broth. Mueller Hinton Agar (MHA) supplemented with 5% defibrinated sheep blood was used to grow *P. multocida* and the liquid medium used was CAMHB. They were incubated in a static incubator at 37 °C for 18-24 h.

Bacterial counts were determined by serial dilution and spot plate counts. Ten-fold or 100-fold dilutions were carried out in phosphate buffered saline. Three 10 µL drops of the appropriate dilutions were dropped onto the agar surface and allowed to dry. After 24 h incubation, the mean CFU count for each 10 µL was determined and multiplied by 100 and then multiplied by the dilution factor to give the original CFU/mL.

Minimum inhibitory and minimum bactericidal concentrations

MICs establish drug concentrations for inhibition of bacterial growth and MBCs determine the concentration to achieve bactericidal effects, defined as a 3log₁₀ reduction in count. MICs and MBCs were determined in broth on 96 well plates by microdilution in accordance with CLSI guidelines for six isolates each of *A. pleuropneumoniae* and *P.*

multocida, except that: (1) five sets of overlapping two-fold serial dilutions of florfenicol were used and (2) determinations were also made in pig serum using five sets of overlapping two-fold dilutions. The bacterial culture was grown to 0.5 McFarland Standard. This was diluted ten-fold to obtain the intended starting inoculum, $1-2 \times 10^7$ CFU/mL, which is higher than the CLSI recommendation of 5×10^5 CFU/mL. This higher inoculum count was selected to be equivalent clinically to a medium to heavy microbial challenge.

Florfenicol solution, media and culture were added sequentially to the wells of 96-well plates. The plates were sealed and incubated statically at 37 °C for 24 h. Spot plate counts were prepared immediately after plate inoculation. Each test on each isolate was undertaken in triplicate. ATCC isolates were used in all assays at the CLSI recommended strength of 5×10^5 CFU/mL. A positive control well contained medium and pathogen only and a negative control well contained medium and florfenicol only. Blank controls contained medium alone. MBCs were determined from the wells examined for growth to determine MIC and for five subsequent concentrations higher than MIC by spot plating.

Mutant prevention concentration

Fresh cultures were grown on agar and approximately 100 single CFUs collected on a sterile swab to inoculate culture from plates to a volumetric flask containing 200 mL of pre-warmed broth. This was placed in a static incubator overnight. Next day, 1 mL of culture was added to 9 mL CAMHB and placed in an orbital incubator for 4 h at 37 °C and 180 rpm. After 4 h, the bacterial suspension yields a theoretical count of $1-2 \times 10^{11}$ CFU/mL. A spot plate was used to confirm inoculum density.

189

190 Drug concentrations used were 1, 2, 4, 8, 16, 32, 64 and 128 multiples of the MIC for
191 each isolate. The concentration ranges were narrowed down two further times. Florfenicol
192 solution (0.5 mL volume) was applied to agar plates. After drying, 100 µL of culture were
193 added and the plate allowed to dry. Plates were incubated at 37 °C for 72 h and checked for
194 growth every 24 h. MPC was the lowest florfenicol concentration inhibiting bacterial growth
195 completely after 72 h incubation. Each experiment was repeated in triplicate for six isolates of
196 each test organism. The method was validated [using a parallel design](#) against that described by
197 Blondeau (2009) and yielded similar results for two isolates of *P. multocida* repeated three
198 times (results not shown).

199

200 *Inoculum size and MIC*

201 Three starting inoculum strengths were tested. A 0.5 McFarland standard was made,
202 providing a theoretical value of 1.5×10^8 CFU/mL (high). Two 1:10 dilutions provided $1.5 \times$
203 10^6 CFU/mL (medium) and two further 1:10 dilutions gave a count of 1.5×10^4 CFU/mL (low).
204 MICs were determined in triplicate for six isolates each of *P. multocida* and *A.*
205 *pleuropneumoniae* in CAMHB and serum.

206

207 *Antimicrobial growth (time-kill) curves*

208 Two to three colonies of the test isolate were added to 5 mL broth or serum and
209 incubated overnight in an orbital incubator, as described above. Fifty µL of culture were diluted
210 1:50 in pre-warmed broth/serum and incubated statically for 1 h at 37 °C to a 0.5 McFarland
211 Standard. This was diluted ten-fold to obtain the intended starting inoculum, consistent with

MICs at 2×10^7 CFU/mL, and confirmed using colony counts. Drug concentrations of 0.25, 0.5, 1, 1.5, 2, 4, 6 and 8 x MIC were prepared in pre-warmed broth or serum. Ten μ L of culture were used to inoculate the dilutions to give a 1 mL final volume. Fifty μ L of each culture were sampled and bacterial count determined by serial dilution and spot plates at nine times: 0, 0.25, 0.5, 0.75, 1, 2, 4, 8 and 24 h incubation. A negative control contained no organism, whilst the positive control contained no drug. Spot plates were examined for contamination and biochemical identification methods were applied. Each test was repeated in triplicate for six isolates of both organisms in broth and serum. The lower limit of quantification (LLOQ) was 33 CFU/mL.

Data collection and analysis

Data were recorded on Microsoft excel and processed using GraphPad Prism v6. The data were analysed using IBM SPSS Statistics 22 by Kruskal-wallis H with post-hoc Mann-Whitney *U* test.

Results

Protein binding

The mean (standard deviation (SD)) protein binding for florfenicol was 64.9 (17.38)% of total concentration for three batches of serum for concentrations over the range 0.1 to 10 μ g/mL. The degree of protein binding was independent of concentration.

Minimum inhibitory, minimum bactericidal and mutant prevention concentrations

Table 1 indicates geometric means for MIC, MBC and MPC in broth and serum. All tests were conducted on six isolates of each species, and each test on each organism was carried out in triplicate. Then, for each triplicate analysis, means were determined. Table 1 shows the means of data for six isolates. For both organisms and all three indices of potency, there were no significant differences between broth and serum. However, for all three indices and both organisms, serum values corrected for protein binding (fu serum MIC) were significantly less than corresponding broth values. After MIC correction to allow for the microbiologically inactive protein bound fraction, the mean fu serum MIC was 0.11 µg/mL, which was 3.82-fold lower than the broth MIC. For the free drug concentration in serum, MBC was 5.75-fold lower than the MBC in broth. For *A. pleuropneumoniae*, MICs were similar in broth (0.38 µg/mL) and serum (0.39 µg/mL). However, again the fu serum MIC (0.14 µg/mL) was 2.71-fold lower than the broth MIC and the fu serum MBC was 2.79-fold lower than the broth MBC.

These differences are clarified in Table 2, which indicates serum:broth ratios and fraction unbound (fu) serum:broth ratios. Differences for all three indices of potency, MIC, MBC and MPC, between broth and fu serum values were statistically significant. Moreover, the amended ratios, fu serum:broth, were broadly similar, 0.37:1, 0.33:1 and 0.45:1 for MIC, MBC and MPC, respectively, for *A. pleuropneumoniae* and corresponding values were 0.27:1, 0.20:1 and 0.25:1 for *P. multocida* (Table 2).

Table 3 presents the data as MBC:MIC and MPC:MIC ratios. The latter were similar for broth and serum, 10.3:1 and 12.5:1, respectively, for *A. pleuropneumoniae*, and 13.4:1 and 13.6:1 for *P. multocida*.

Individual isolate MIC, MBC and MPCs for six isolates each of *A. pleuropneumoniae* and *P. multocida* in broth and serum are presented in Supplementary Figs. 1, 2 and 3. Further investigations of the effect of pH, cation adjustments and serum/broth mixtures on MIC were undertaken and are presented in Supplementary Figs. 4, 5, 6 and 7.

Effect of inoculum size on MIC

Tables 4 and 5 present mean (SD) MICs for inocula of three strengths, together with high:medium, high:low and medium:low MIC ratios for six isolates each of *A. pleuropneumoniae* and *P. multocida*. For *P. multocida*, high:low ratios were 5.3:1 (broth) and 5.4:1 (serum) and were statistically different ($P < 0.01$). High:medium MIC ratios were 2.6:1 and 2.8:1 and were statistically different ($P < 0.05$) for broth and serum, respectively. For *A. pleuropneumoniae*, high:low MIC ratios were 6.0:1 (broth) and 9.4:1 (serum) (broth ratio $P < 0.05$, serum ratio $P < 0.01$), whilst high:medium ratios were 2.6:1 and 2.9:1 for broth and serum, respectively ($P < 0.05$ for both).

Time-kill curves

Time-kill curves for florfenicol are illustrated in Figs. 1 (*A. pleuropneumoniae*) and 2 (*P. multocida*), where 0, -3 and -4 $\log_{10}$ differences in CFU/mL are equivalent, respectively, to bacteriostatic, bactericidal and 10,000-fold reduction in count effects. For *A. pleuropneumoniae*, little inhibition occurred at concentrations less than 1 x MIC for both media. A bactericidal effect was achieved at 2 x MIC by 8 h in broth, and at 4 x MIC by 8 h in

serum. Virtual eradication (count less than 33 CFU/mL) was obtained after 24 h at 6 and 8 x MIC in both broth and serum.

For *P. multocida*, both matrices by 4 h, 4 x MIC produced a bactericidal effect, 6 x MIC demonstrated greater than 4 log₁₀ decrease in count and for 4-8 x MIC there was virtual eradication in broth at 24 h. For serum, virtual eradication was achieved at 24 h with 6 and 8 X MIC. There was no re-growth at 24 h. The action of florfenicol in both fluids for both species was classed as concentration-dependent.

Discussion

Some authors recommend restriction of the term MIC to measurements made in broths prescribed in CLSI and EUCAST guidelines. However, MIC is a widely accepted and understood term, even when growth matrices other than those required to meet CLSI and EUCAST standards have been used. For example, MIC was used by Honeyman et al. (2015) to compare potencies of several tetracyclines in broth and a 50% broth:50% serum mixture. The differences obtained could not be accounted for solely by the degree of drug-protein binding. Furthermore, Zeitlinger et al. (2011) has commented that “bacteria with appropriate and well-defined growth in the selected medium should be employed....in order to be able to extrapolate data from various models to in vivo situations, models should always attempt to mimic physiological conditions as closely as possible”. These considerations relate to accuracy of MIC determination; this can generally be obtained from broth MIC results by applying a robust scaling factor to bridge in vitro MHB to in vivo relevant serum/plasma/blood values.

For porcine pneumonia, it would be theoretically desirable to monitor drug potency by measuring MIC in pulmonary epithelial lining fluid (PELF). However, collection of cell-free PELF in sufficient volumes for MIC determinations on a reasonable number of isolates would be challenging. Therefore, if one determines serum MIC values and corrects them for protein bound (inactive) drug fraction, this might be preferable to use of virtually protein-free broths for dose prediction purposes, for some drug classes, such as macrolides/tiamidolides, as shown for tulathromycin (Toutain et al., 2016). This is because, at steady state equilibrium, the free serum concentration will be the same as the free biophase concentration. Moreover, drug activity in serum is much closer to activity in broth from a biological fluid composition perspective. Thus, serum (but not broths) is likely to have concentrations of electrolytes, urea, leucocytes, glucose etc. closer to the biophase than do broths (Zeitlinger et al., 2011). Hence, whilst serum is not identical to the biophase matrix, it is likely to be closer to the biophase in chemical composition than broths, and indeed in respect of some immunological components also. Brentnall et al. (2012) reported on the differing chemical compositions of Mueller Hinton Broth and calf serum. Of course, the in vitro data presented in this paper should be now be used in dose estimation studies, based on PK/PD modelling, and these doses, in turn, will require confirmation (or not) in disease model studies and clinical trials.

The mean percentage protein binding of florfenicol in pig serum, over the therapeutic concentration range of 0.1 to 10 µg/mL, was 64.9%. This is higher than the binding reported for other species; 17.8% for calf serum (Illambas et al., 2013) 26.6% in the goat (Atef et al., 2001) and 18.1-21.1% in Muscovy ducks (El-Banna, 1998). These differences impact on dose schedules for clinical use.

If the only potency difference between broth and serum for antimicrobial drugs is attributable to protein binding in the latter fluid and the virtual absence of protein (notably albumin) in most broths, the correction for binding would yield serum:broth ratios of 1:1 for each index. In this study, the fu serum:broth MIC, MBC and MPC ratios were 2.71:1, 3.82:1 and 3.10:1 (*A. pleuropneumoniae*) and 5.75:1, 2.27:1 and 3.99:1 (*P. multocida*) respectively. These ratios reflect the correction, which is routinely made to allow for the fact that protein bound drugs are microbiologically inactive (Merrikin et al., 1983; Zeitlinger et al., 2004). These experimental values were significantly different from the predicted 1:1 ratio. Therefore, the potency in serum exceeded that in broth and the former may be a more relevant index for linking with pharmacokinetic data to predict dose schedules for clinical use. Indeed, the present findings indicate that use of serum MIC data would generate estimated doses some three to four times lower than those predicted by broth MICs and, if calculated doses were based on MBCs, they would be 3 to 6 times lower.

These serum/broth differences emphasise the importance not only of comparing potency in several matrices (e.g. differing broths, serum/plasma, inflammatory exudate) but also of acquiring larger data sets in these fluids. Nevertheless, this study with a small number of isolates for each bacterial species quantified inter-isolate variability in the antimicrobial action of florfenicol against two bacterial pathogens, *A. pleuropneumoniae* and *P. multocida*, for four indices of activity in two matrices, broth and serum. These findings on serum/broth differences should be extended also to other pathogenic species, as potency is a factor determining dose and differences are likely to be matrix, drug, species and strain dependent. In future studies, it will be important to determine if sera from different breeds/environmental conditions/continents/countries/disease states/ages etc. will lead to differing results in potency,

as monitored by indices such as MIC, MBC and MPC. Therefore, it will be necessary to ensure standardisation across laboratories. This paper is no more than a first step in this direction.

To achieve clinical and bacteriological efficacy, as well as minimising the emergence of resistance, an important consideration is pathogen load. For prophylaxis, metaphylaxis and treatment early in the course of disease, the bacterial pathogen load may be small or absent. Many drugs will then prevent or cure disease at low dose rates, in association with natural body defences. The major challenge, however, is to achieve a bacteriological cure, when pathogen density in the biophase is high. In this study, pathogen load affected significantly MICs in both broth and serum and for both species investigated. However, it should be noted that florfenicol is not licensed for prophylactic use.

De Jong et al. (2014) documented florfenicol MIC distribution frequencies for *A. pleuropneumoniae* and *P. multocida* for EU pig isolates between 2002 and 2006. The MICs for *P. multocida* isolates were 0.25 µg/mL (72/230), 0.5 µg/mL (155/230) and 1 µg/mL (2/230). The data, valid for the objectives set, were obtained using CLSI methodologies and therefore did not use five overlapping sets of two-fold dilutions, nor take account of serum/broth differences. Nevertheless, their broth MIC values are of a similar order of magnitude to those reported in this study.

The mean broth MPCs in this study were 3.84 and 5.63 µg/mL for *A. pleuropneumoniae* and *P. multocida*, respectively. These are comparable with may be compared data for 285

cattle isolates of *M.haemolytica*: the MPC₉₀ was 8 µg/mL and the MIC₅₀ was 4 µg/mL (Blondeau et al., 2012).

Pharmacokinetic data for florfenicol in pigs administered intramuscularly at 10 mg/kg, using licensed products, can be linked with the serum potency data generated in this study (Liu et al., 2003). The maximum in vivo serum concentration (free plus bound) was 3.2 µg/mL, whilst mean serum MICs and MPCs were 0.39, 4.89 µg/mL (*A. pleuropneumoniae*), 0.30, and 4.08 µg/mL (*P. multocida*). The comparison indicates that the currently recommended florfenicol dose provides concentrations above MICs for at least 48 h but MPC was not attained, even at the maximum in vivo concentration. Therefore, a dose to ensure avoidance of resistance emergence may not be quite attainable.

For *A. pleuropneumoniae* and *P. multocida* the range of eight multiples of MIC, 0.25 to 8 x MIC, was sufficient to establish bacteriostatic, bactericidal and virtual eradication effects at 24 h and to plot the time course of killing pattern at earlier times. This was rapid for multiples of 4 x MIC and greater for both *A. pleuropneumoniae* and *P. multocida*. The killing action was classified as concentration dependent.

For future studies, refinements of the methodology used in this investigation would be to establish time-kill curves not only in serum but also in the presence of other "natural" constituents, such as antibodies and leucocytes. The present time-kill studies were based on the widely accepted exposure of the bacteria to fixed florfenicol concentrations for a fixed time period. In vivo, concentrations in both serum and the biophase first increase and then decrease

after systemic, non-vascular dosing. Therefore, In vitro time-kill techniques, such as hollow fibre models of growth inhibition, which better reflect the circumstances of clinical exposure, could be used in future studies to simulate in vivo concentration-time profiles.

Nightingale and Murakawa (2002) noted a greater potency of some macrolides in serum compared to broth and they recommended use of the former fluid to generate data relevant to clinical in vivo circumstances. Mitchell et al. (2012, 2013) reported markedly greater potency of the triamilides, tulathromycin and gamithromycin, in in vitro MIC and time-kill studies for the calf pathogen *Mycoplasma mycoides mycoides* small colony. Likewise, Godinho et al. (2005) and Godinho (2008) reported 8-fold reductions in MIC for both *M. haemolytica* and *P. multocida* of calf origin, when broth was supplemented with 40% calf serum, compared to 100% broth MICs. In extension of this work, comparing 100% serum with 100% broth, Toutain et al. (2016) reported MIC broth:serum MIC ratios of 47:1 and 53:1, respectively, for these two pathogens. Differences were far greater than could be accounted for by correction of serum MICs for protein binding, and indicated potentiation of the action of tulathromycin by some serum factor. In stark contrast, Brentnall et al. (2012, 2013b) obtained much higher MICs in serum compared to broth for oxytetracycline and, even with correction for protein binding, the difference was 6-fold. These and the present data indicate drug specific properties interacting with growth matrix; one possibility is differing rates of logarithmic growth of bacteria in broths and serum, potentially leading to different levels of challenge. Additionally, for some drug classes e.g. tetracyclines, several actions have been reported that may influence clinical outcome, including immunomodulatory and anti-inflammatory properties (Pasquale & Tan, 2005; Woo et al., 1999).

Conclusions

Matrix dependent effects on quantitative MIC, MBC and MPC values were obtained for florfenicol; after correction for binding to serum protein, all three indices of potency were significantly lower in serum than in broth, indicating that correction for protein binding (whilst necessary) is not sufficient to account for matrix potency differences. Time-kill data demonstrated a co- or concentration-dependent killing action in both growth matrices. It is the hypothesis of this paper that in vitro plasma/serum concentration of florfenicol is more likely to be close to the in vivo concentration required for efficacy than potency estimated in broth. Differences between broth and porcine serum may have implications for optimization of dosage schedules for clinical use.

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Conflict of interest statement

None of the authors of this paper have a financial or personal relationship with other people or organization that could inappropriately influence or bias the content of the paper. During the last 5 years the authors interests have included the following: P. Lees (consultancy advice supplied to Bayer Animal Health, Norbrook Laboratories Ltd. and Pfizer Animal Health); and S. Hobson (employee of Norbrook Laboratories Ltd.).

Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:
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Table 1

A. pleuropneumoniae (APP) and *P. multocida* (PM) MIC, MBC and MPC for broth, serum and unbound fraction serum concentrations (fu serum)^a.

Organism	Medium	MIC	MBC	MPC
APP	Broth	0.38 (0.11)	2.79 (0.54)	3.84 (0.70)
	Serum	0.39 (0.17)	2.61 (0.83)	4.89 (1.93)
	fu Serum	0.14 (0.06)*	0.92 (0.29)*	1.71*
PM	Broth	0.42 (0.13)	1.38 (1.23)	5.63 (1.70)
	Serum	0.30 (0.16)	0.69 (0.67) *	4.08 (1.69)
	fu Serum	0.11 (0.06)*	0.24 (0.08)*	1.43*

^aGeometric means (SD) of florfenicol concentrations: triplicate analyses for each of six isolates of each organism ($n=6$). Significance of differences between broth and fu serum: * $P < 0.01$. MIC = minimum inhibitory concentration, MBC = minimum bactericidal concentration, MPC = mutant prevention concentration.

Table 2

A. pleuropneumoniae (APP) and *P. multocida* (PM) serum:broth and unbound fraction serum concentration (fu serum):broth ratios^a for MIC, MBC and MPC

Organism	Medium	MIC	MBC	MPC
APP	Serum:broth	0.96:1	1.07:1	1.27:1
	fu Serum:broth	0.37:1	0.33:1	0.45:1
PM	Serum:broth	0.72:1	0.50:1	0.73:1
	fu Serum:broth	0.27:1	0.20:1	0.25:1

^aRatios for triplicate analyses for each of six isolates of each organism ($n=6$). MIC = minimum inhibitory concentration, MBC = minimum bactericidal concentration, MPC = mutant prevention concentration.

Table 3

A. pleuropneumoniae (APP) and *P. multocida* (PM) MBC:MIC and MPC:MIC ratios^a for broth and serum

Organism	Medium	MBC:MIC	MPC:MIC
APP	Broth	7.36 : 1	10.32 : 1
	Serum	6.64 : 1	12.47 : 1
PM	Broth	3.26 : 1	13.41 : 1
	Serum	2.26 : 1	13.60 : 1

^aTriplicate analyses for each of six isolates of each organism ($n=6$). MIC = minimum inhibitory concentration, MBC = minimum bactericidal concentration, MPC = mutant prevention concentration.

Table 4

P. multocida (PM) MICs for broth and serum with three inoculum sizes^a, serum:broth ratios and inoculum size ratios

Inoculum size	Broth MIC	Serum MIC	Serum:broth ratio
High	0.78 (0.19)	0.73 (0.16)	0.94 : 1
Medium	0.30 (0.10)	0.26 (0.02)	0.86 : 1
Low	0.15 (0.04)	0.13 (0.04)	0.92 : 1
High:medium ratio	2.6 : 1 ¹	2.8 : 1 ³	
High:low ratio	5.3 : 1 ²	5.5 : 1 ⁴	
Medium:low ratio	2.1 : 1	1.9 : 1	

^aInoculum size: high (10⁸ CFU/mL), medium (10⁶ CFU/mL) and low (10⁴ CFU/mL). Minimum inhibitory concentration (MIC) values are geometric means (SD) for triplicate analyses for 6 isolates (*n*=6). Differences in MIC ratios between inoculum sizes: *P* = ¹0.03, ²0.01, ³0.03, ⁴0.01.

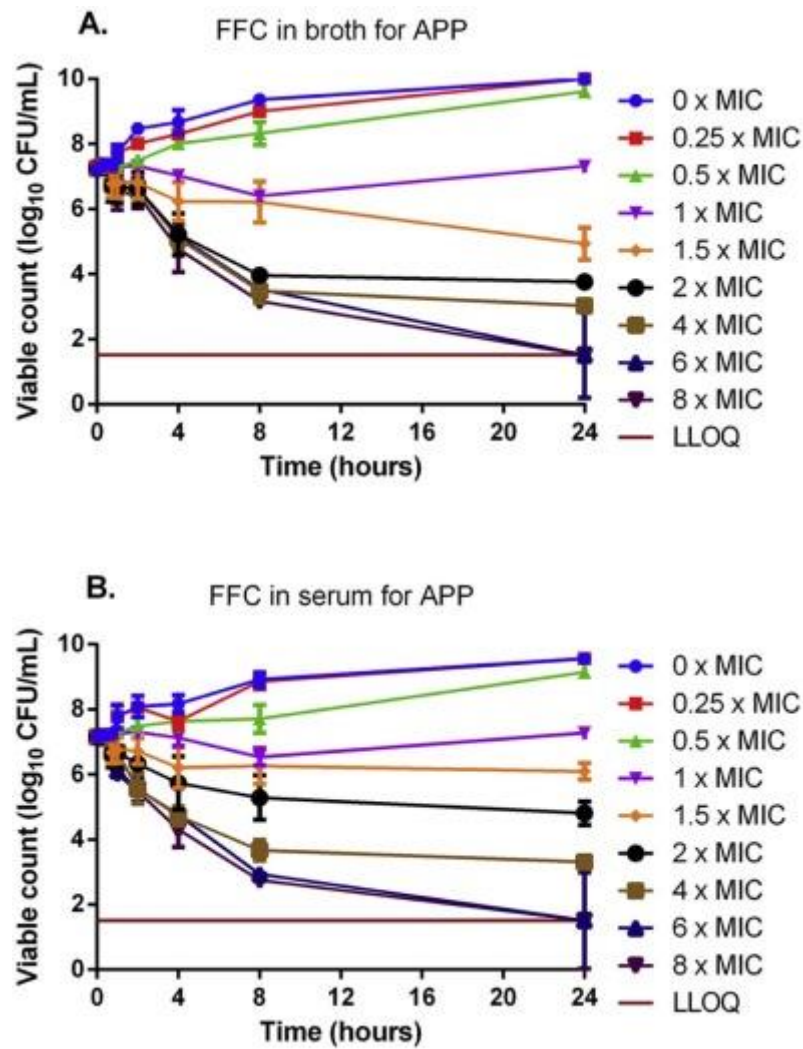
Table 5

A. pleuropneumoniae (APP) MICs for broth and serum with three inoculum sizes^a, serum:broth ratios and inoculum size ratios

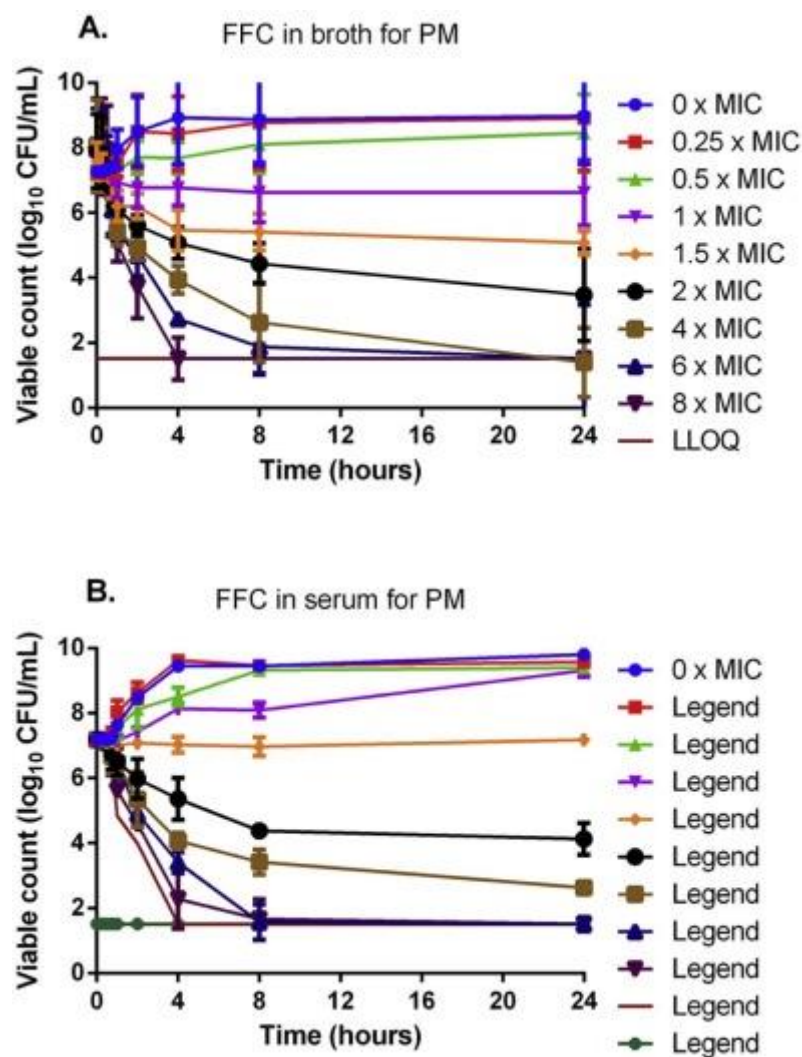
Inoculum size	Broth MIC	Serum MIC	Serum:broth ratio
High	0.81 (0.22)	1.30 (0.55)	1.62 : 1
Medium	0.32 (0.10)	0.44 (0.04)	1.40 : 1
Low	0.13 (0.02)	0.14 (0.01)	1.05 : 1
High:medium ratio	2.6 : 1 ¹	2.9 : 1 ³	
High:low ratio	6.0 : 1 ²	9.4 : 1 ⁴	
Medium:low ratio	2.4 : 1	3.2 : 1	

^aInoculum size: high (10⁸ CFU/mL), medium (10⁶ CFU/mL) and low (10⁴ CFU/mL). Minimum inhibitory concentration (MIC) values are geometric means (SD) for triplicate analyses for six isolates (*n*=6). Differences in MIC ratios between inoculum sizes: *P* = ¹0.03, ²0.02, ³0.03, ⁴0.01.

Figure 1. In vitro inhibition of growth of *A. pleuropneumoniae* over 24 h exposure to 8 multiples of MIC in (A) Columbia broth supplemented with NAD and (B) pig serum ($n = 6$). Bars indicate standard deviation. LLOQ= 33 CFU/mL.



638 **Figure 2.** In vitro inhibition of growth of *P. multocida* over 24 h exposure to 8 multiples of
 639 MIC in (A) CAMHB and (B) pig serum ($n = 6$). Bars indicate standard deviation. LLOQ = 33
 640 CFU/mL.



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