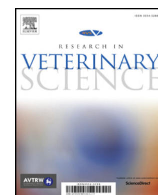
Research in Veterinary Science 111 (2017) 43–48

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc



Potency of marbofloxacin for pig pneumonia pathogens *Actinobacillus pleuropneumoniae* and *Pasteurella multocida*: Comparison of growth media

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ARTICLE INFO

Article history:
Received 11 May 2016
Received in revised form 27 September 2016
Accepted 1 November 2016
Available online xxxx

Keywords:
Marbofloxacin
Minimum Inhibitory Concentration
Minimum Bactericidal Concentration
Mutant Prevention Concentration
Pasteurella multocida
Actinobacillus pleuropneumoniae

ABSTRACT

Pharmacodynamic properties of marbofloxacin were established for six isolates each of the pig respiratory tract pathogens, *Actinobacillus pleuropneumoniae* and *Pasteurella multocida*. Three *in vitro* indices of potency were determined; Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Mutant Prevention Concentration (MPC). For MIC determination Clinical Laboratory Standards Institute guidelines were modified in three respects: (1) comparison was made between two growth media, an artificial broth and pig serum; (2) a high inoculum count was used to simulate heavy clinical bacteriological loads; and (3) five overlapping sets of two-fold dilutions were used to improve accuracy of determinations. Similar methods were used for MBC and MPC estimations. MIC and MPC serum:broth ratios for *A. pleuropneumoniae* were 0.79:1 and 0.99:1, respectively, and corresponding values for *P. multocida* were 1.12:1 and 1.32:1. Serum protein binding of marbofloxacin was 49%, so that fraction unbound (fu) serum MIC values were significantly lower than those predicted by correction for protein binding; fu serum:broth MIC ratios were 0.40:1 (*A. pleuropneumoniae*) and 0.50:1 (*P. multocida*). For broth, MPC:MIC ratios were 13.7:1 (*A. pleuropneumoniae*) and 14.2:1 (*P. multocida*). Corresponding ratios for serum were similar, 17.2:1 and 18.8:1, respectively. It is suggested that, for dose prediction purposes, serum data might be preferable to potency indices measured in broths.

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1. Introduction

Internationally accepted methods, guidelines and standards for Minimum Inhibitory Concentration (MIC) determination have been set by the European Union Committee on Antimicrobial Sensitivity testing (EUCAST) and the Clinical Laboratory Standards Institute (CLSI, 2008). These standards provide the advantage of consistency in comparing MICs between individual experimenters, laboratories and across countries (Papich, 2013). For MIC determination, EUCAST and CLSI require the use of two-fold dilutions. 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. CLSI reports microbiological Cut-Offs (CO_{WT}) and EUCAST reports Epidemiological Cut-Offs (ECOFF). These are often identical but differences occur for some drugs.

Despite these clear benefits, for the purposes of the present study, the CLSI/EUCAST methods of determining MIC have two disadvantages. First, being based on two-fold dilutions, there is potential for up to 100% error on single isolate estimates, thus having a limitation regarding accuracy for a small number of isolates. To partially meet this concern, previously we have used five sets of overlapping two-fold dilutions; this reduces inaccuracy from approaching 100% to not exceeding 20% (Aliabadi and Lees, 2001; Sidhu et al., 2010). Second, the CLSI/EUCAST standards are based on the use of broths, specifically formulated to facilitate bacterial growth *in vitro*. They differ in composition from biological fluids and hence may not reflect bacterial growth conditions *in vivo*. To enable comparisons between broths and biological fluids as growth matrices, and to evaluate possible differences between them, previous authors have used serum, plasma and inflammatory exudate (Aliabadi and Lees, 2001, 2002; Nightingale and Murakawa, 2002; Zeitlinger et al., 2004, 2008; Sidhu et al., 2010).

To optimise clinical efficacy and minimise the emergence of resistance to antimicrobial drugs, a third consideration is dosage required for differing pathogen loads. For metaphylaxis and treatment early in the course of disease, when the pathogen load is absent or low, many drugs will either prevent or cure disease, acting in support of natural body defences. However, this general consideration does not apply to marbofloxacin, which is recommended solely for therapeutic use. The major challenge for antimicrobial drugs is to select a dosage regimen which provides a bacteriological cure and avoids the emergence of resistance, when pathogen numbers in the biophase are high (Mouton et al., 2011a,b; Martinez et al., 2012; Papich, 2014). For this reason, a high starting inoculum count of approximately 10^7 CFU/mL was

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http://dx.doi.org/10.1016/j.rvsc.2016.11.001
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selected for use in this study, in preference to the inoculum count of 5×10^5 CFU/mL recommended in CLSI and EUCAST guidelines.

The product literature for marbofloxacin contains the statement, "Official and local antimicrobial policies should be taken into account when the product is used. Fluoroquinolones should be reserved for the treatment of clinical conditions which have responded poorly, or are expected to respond poorly, to other classes of antimicrobials. Whenever possible, fluoroquinolones should only be used based on susceptibility testing. Use of the product deviating from the instructions given in the SPC/datasheet may increase the prevalence of bacteria resistant to the fluoroquinolones and may decrease the effectiveness of treatment with other quinolones due to the potential for cross resistance."

The aims of this investigation were: (1) to determine the degree of protein binding of marbofloxacin in pig serum; (2) for marbofloxacin and six isolates each of two pig respiratory pathogens (*A. pleuropneumoniae* and *P. multocida*) to determine three indices of potency, MIC, Minimum Bactericidal Concentration (MBC) and Mutant Prevention Concentration (MPC) using five sets of overlapping two-fold dilutions and to compare each index in two matrices, CLSI recommended broths and pig serum.

2. Materials and methods

2.1. Marbofloxacin serum protein binding

Marbofloxacin concentration in pig serum *in vitro* was determined by high pressure liquid chromatography (HPLC) (Aliabadi and Lees, 2002). The HPLC system comprised a Dionex Ultimate 3000 pump and autosampler connected to a Dionex RF 2000 fluorescence detector (Thermo Fisher UK Ltd., Hemel Hempstead, UK). Fluorescence detection was set at an excitation wavelength of 295 nm and an emission wavelength of 500 nm. Chromatographic data were analysed using Chromeleon and concentrations of marbofloxacin were calculated using ratios of peak area marbofloxacin:internal standard. Marbofloxacin concentrations used were 0, 0.0025, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 μg/mL, incubated for 30 min. Two sample sets were used: (1) spiked marbofloxacin standards in serum to determine total concentration; (2) an aliquot of these serum samples filtered using ultra-filtration devices (Amicon Ultra Centrifugal filters, Ultracel 10 k, Sigma-Aldrich Ltd., Dorset, UK). The 3 mL sample aliquot was placed in the ultra-filter unit and centrifuged at 4000 ×*g* for 20 min at 25 °C. The ultra-filtrate was harvested from the reservoir of the system and assayed to determine concentration in the protein free fraction. For each concentration, determinations were made on three batches of pig serum.

$$\%\ \text{Protein Binding} = \frac{[total - unbound] \times 100}{Total}$$

2.2. Bacterial isolates

Twenty isolates of *P. multocida* were supplied by Don Whitley Scientific (Shipley, West Yorkshire, UK). They also supplied three ATCC reference strains for use in MIC tests; *A. pleuropneumoniae* ATCC 27090, *Enterococcus faecalis* ATCC 29212 and *E. coli* ATCC 25922. Eight isolates of *A. pleuropneumoniae* were supplied by A. Rycroft (Royal Veterinary College, Herts., UK). All *A. pleuropneumoniae* and *P. multocida* isolates were derived from EU field cases of pig pneumonia. They were stored at −80 °C in 10% Marvel® milk powder, 15% glycerol in sterile distilled water. 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) ability to grow logarithmically in both CLSI recommended broth and pig serum; (2) susceptibility to marbofloxacin, as indicated by MIC determined using doubling dilutions; and (3) selection of isolates with the highest and lowest broth MICs plus four isolates with intermediate MICs.

2.3. Culture methods and bacterial counts

For *A. pleuropneumoniae*, Chocolate Mueller Hinton Agar (CMHA) was used for growth on a solid medium and Columbia broth supplemented with 2 μg/mL nicotinamide adenine dinucleotide (NAD) was the liquid broth. Mueller Hinton agar supplemented with 5% defibrinated sheep blood (MHA) was used to grow *P. multocida* and the liquid medium was Cation Adjusted Mueller Hinton Broth (CAMHB). Organisms 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 for 10 min before incubating for 24 h. The mean CFU count for each 10 μL was determined, multiplied by 100 and then multiplied by the dilution factor to obtain the initial CFU/mL.

2.4. Minimum Inhibitory and Minimum Bactericidal Concentrations

MICs were determined by microdilution for six isolates each of *A. pleuropneumoniae* and *P. multocida*, in accordance with CLSI guidelines, except for: (a) using five sets of overlapping two-fold serial dilutions to increase accuracy; (b) making determinations in serum as well as broth; and (c) growing cultures to 0.5 McFarland Standard (approximately 1–2×10^8 CFU/mL) and this was diluted ten-fold to obtain a starting inoculum of 2×10^7 CFU/mL. This is higher than, and therefore also a deviation from the CLSI guidelines, which recommend a starting count of 5×10^5 CFU/mL. The higher count was selected to provide a medium to heavy microbial load.

Marbofloxacin, media and culture were added successively to each well of 96-well plates. Plates were sealed and incubated statically at 37 °C for 24 h. Spot plate counts were prepared immediately after plate inoculation. Tests on each isolate were undertaken in triplicate. Control ATCC isolates were used at a count of 5×10^5 CFU/mL as per CLSI guidelines. A positive control well contained medium and pathogen only and a negative control contained medium and marbofloxacin solution. Blank controls contained medium only. For MBC, wells were examined for growth to determine MIC and, in addition to that well, five subsequent concentrations higher than MIC were examined by spot plating. This indicates a $3\log_{10}$ reduction in inoculum count.

2.5. Mutant Prevention Concentration

After growing fresh cultures on agar, approximately 100 single colony forming units (CFU) were used to inoculate culture from plates into a volumetric flask containing 200 mL of pre-warmed broth. This was incubated statically overnight at 37 °C. Next day, 1 mL of culture was added to 9 mL of broth and placed in an orbital incubator for 4 h at 37 °C and 180 rpm. After 4 h, the bacterial suspension yields 1–2×10^{11} CFU/mL. A spot plate was used to confirm inoculum density.

Final drug concentrations were 1, 2, 4, 8, 16, 32, 64 and 128 multiples of MIC for each of six isolates of each species. These ranges were narrowed down two further times, for example if MPC was 64×MIC the next range would be 32, 36, 40, 44, 48, 52, 56, 60 and 64×MIC, and the final range if the MPC was 36×MIC would be 32, 32.5, 33, 33.5, 34, 34.5, 35, 35.5 and 36×MIC. Five hundred microliters of marbofloxacin solution was applied to cold, dry agar plates and left to dry. Culture (100 μL) was added to the plate and allowed to dry. Plates were incubated at 37 °C for 72 h and checked for growth every 24 h. MPC was the lowest marbofloxacin concentration inhibiting bacterial growth completely after 72 h incubation.

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