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1 **Determination of minimum inhibitory and minimum bactericidal concentrations of**
2 **tiamulin against field isolates of *Actinobacillus pleuropneumoniae***

3

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17

18 **ABSTRACT**

19

20 Tiamulin activity was measured against 19 UK field isolates of *A. pleuropneumoniae*

21 collected between 2003 and 2009 and the type strain ATCC 27090 as a control, with the

22 intention of comparing broth with serum as growth media. Broth microdilution MIC/MBC

23 tests were performed in accordance with the Clinical and Laboratory Standards Institute

24 (CLSI) guideline M31-A3, in 'Veterinary Fastidious Medium' (VFM) (supplemented

25 Mueller-Hinton broth at pH 7.3) and in 100% swine serum. For improved precision, a

26 modified, overlapping doubling-dilution series was used (tiamulin concentration range 0.3-72

27 µg/ml). The MBC was reported as the lowest concentration producing a 99.9% reduction in

28 bacterial density in the sub-cultured well contents, relative to the starting inoculum. The
29 mean MBC/MIC ratio for tiamulin against *A. pleuropneumoniae* in VFM was low (1.74: 1),
30 even though tiamulin is classed as a bacteriostatic drug. Only three of the 19 isolates and the
31 reference strain grew in 100% serum and their MICs were higher than those determined in
32 VFM. It is postulated that this difference was due to differences in pH of the matrices or
33 binding of tiamulin to serum proteins or a combination of both factors.

34

35 **Keywords:**

36 *Actinobacillus pleuropneumoniae*

37 PK/PD

38 Tiamulin

39 MIC

40 MBC

41 Serum culture

42

43

44

45 **1. Introduction**

46 Serum/plasma concentrations linked to indices such as MIC are well-established surrogates
47 for most antimicrobial drugs. However, correlating the pharmacokinetics (PK) of tiamulin
48 hydrogen fumarate concentrations in plasma with the pharmacodynamics (PD) measured as
49 minimum inhibitory concentration (MIC) against *Actinobacillus pleuropneumoniae* has been
50 unsuccessful. Using standardized Mueller-Hinton broth (MHB), MIC determinations based
51 on the Clinical and Laboratory Standards Institute (CLSI) methods provided a susceptibility
52 breakpoint ≤ 16 $\mu\text{g/ml}$ against *A. pleuropneumoniae*. This value is much higher than the

53 plasma concentration achieved with therapeutic dose rates of tiamulin, suggesting that either
54 the MIC method of determination in artificial media does not reflect growth conditions in
55 biological fluids such as plasma or circulating concentrations of tiamulin differ significantly
56 from those at the site of infection. High intracellular concentrations of tiamulin in the lung
57 (Anderson *et al.*, 1994; McKellar *et al.*, 2004) and human polymorphonuclear leucocytes
58 (Nielsen and Szancer, 1998) were reported in comparison with plasma concentrations; both
59 tissues: plasma concentration ratios exceeding 18:1. In an artificial *A. pleuropneumoniae*
60 infection study in pigs, an isolate with a tiamulin MIC of 4µg/ml was effectively treated, both
61 clinically and bacteriologically, with 180 ppm tiamulin in the drinking water (Schultz *et al.*,
62 1984; Burch and Klein, 2008). At this dosage, plasma concentrations of approximately
63 0.47µg/ml would be obtained, *i.e.* 1/8th of the MIC.

64 Studies using 2.5% serum in the culture medium (Godinho *et al.*, 2005) and 100% serum
65 (Illambas *et al.*, 2009) reduced the MIC values for tulathromycin against a variety of bacterial
66 respiratory pathogens of bovine origin but in particular *Pasteurella multocida* by 8 and 50
67 times, respectively, and against *A. pleuropneumoniae* (Godinho *et al.*, 2005) by 32 fold. The
68 aims of this study were (1) to determine MICs, Minimum Bactericidal Concentrations
69 (MBCs) and MBC: MIC ratios for tiamulin against *A. pleuropneumoniae* using overlapping
70 sets of doubling dilutions to improve accuracy and (2) to determine whether the MIC and
71 MBC of tiamulin against *A. pleuropneumoniae* were reduced substantially by culturing the
72 organism in 100% swine serum in comparison with MHB.

73

74 **2. Materials and methods**

75 Tiamulin hydrogen fumarate (Denagard® - Novartis Animal Health Inc.) activity was
76 measured against 19 field isolates and one control type strain (ATCC 27090) of *A.*
77 *pleuropneumoniae*. All field strains were isolated during the period 2003-2009, from pigs

78 exhibiting clinical signs of pleuropneumonia, and were identified by veterinary diagnostic
79 laboratories. Following initial isolation, each strain was stored in a cryoprotective suspension
80 at -80°C and was subjected to no more than three subcultures from the original isolate. Strain
81 identification was confirmed before commencing the current study, on the basis of
82 morphological observations.

83 Broth microdilution MIC tests were performed using five overlapping sets of double-
84 dilutions of tiamulin (to improve accuracy of the determinations) (range 0.3 to 72 µg/ml;
85 Figure 1) in accordance with the CLSI guideline M31-A3, using “Veterinary Fastidious
86 Medium” (VFM); (Mueller-Hinton Broth supplemented with 5% lysed horse blood, yeast
87 extract & yeast concentrate), without added serum (exact CLSI method) and in 100% swine
88 serum (supplied frozen by First Link UK Ltd., Birmingham, UK). The MIC plates were
89 incubated in 5% CO₂ for 24 -48h. The MBC of tiamulin against each isolate was reported as
90 the lowest concentration producing a 99.9% reduction in bacterial viable count in the sub-
91 cultured well contents, relative to the initial inoculum, which was enumerated immediately
92 after inoculation by subculturing the positive control well on to Mueller-Hinton agar plates
93 and counting the colonies.

94

95 **3. Results**

96 The MIC and MBC data for 19 field isolates and the reference strain were determined
97 following growth in VFM (Table 1). Only three field isolates and the reference strain grew in
98 100% serum and there were moderate differences in MIC between VFM and serum for these
99 four strains; MICs were 12/24, 14/24, 12/32 and 12/24 µg/ml (mean 12.5/26 µg/ml) for VFM
100 and serum, respectively.

101 For the 19 strains grown in VFM the MIC₅₀ and MIC₉₀ were 12 and 14 µg/ml, respectively,
102 and the MBC₅₀ and MBC₉₀ were 18 and 38 µg/ml, respectively. The MBC/MIC ratio for all

103 20 isolates was 1.74: 1 (Table 2). The pH of the serum was 7.14 and that of the VFM
104 approximately 7.3 (range 7.29 - 7.33).

105

106 **4. Discussion**

107 The MIC values of the isolates grown in VFM were in accordance with previously published
108 data (Jones *et al*, 2002; Matter *et al*, 2006). The mean MBC was relatively low,
109 approximately twice (1.74 times) the MIC for tiamulin against *A. pleuropneumoniae*. This
110 was unexpected, as tiamulin is classified primarily as a bacteriostatic drug, so that a wider
111 ratio might have been predicted. Tiamulin has been shown to inhibit protein synthesis by
112 binding to the 50S ribosomal subunit. It is a strong inhibitor of peptidyl transferase and
113 interacts with domain V of 23S RNA, functionally inhibiting the correct positioning of
114 tRNAs for peptide transfer (Poulsen *et al*, 2001). There is no reason to suppose that this mode
115 of action would be modified or downregulated *in vivo*, in comparison with *in vitro* and such
116 change is unlikely to explain the PK/PD discrepancy.

117 The low MBC/MIC ratio is in accordance with the clinical efficacy in an artificial challenge
118 study (Schultz *et al*, 1984; Burch and Klein, 2008), in which high doses of tiamulin
119 administered via the drinking water (180ppm) achieved a bacteriological cure, as indicated by
120 the inability to isolate *A. pleuropneumoniae* from the lungs. Lower doses of 60 and 120ppm
121 did not achieve bacteriological cure. However, this finding does not resolve the lack of
122 correlation between plasma concentration and MIC/MBC on the one hand and clinical
123 outcome on the other.

124 The growth of *A. pleuropneumoniae* isolates in 100% serum was limited, only 4 of 19 strains
125 showing visible growth. For these 4 strains the MICs were not reduced, as previously
126 observed with 50% serum and 2.5 and 15% serum had no effect on MICs. In fact, MICs were
127 twice as high in serum as in VFM in the present study. Conventionally, (e.g. CLSI standards),

128 MIC determinations are performed using a doubling dilution series of the antimicrobial
129 agents. In that case, a doubling of the MIC is demonstrated by only a single dilution shift in
130 the series and is thus regarded as being within the normal variability of the method. In the
131 present study, we used a dilution series with narrower intervals and the MIC increase in
132 serum is clearly genuine because it corresponds to a shift of 5 dilution steps in the series.
133 Moreover, the lower MICs in VFM could be predicted from the higher pH of that medium,
134 because the slightly more alkaline pH of VFM will lead to a higher unionised: ionised ratio of
135 the drug. Another potentially significant factor is degree of protein binding of tiamulin, which
136 was not measured in this study but has been reported at approximately 40% in swine serum at
137 these concentrations (Ulrich Klein, personal communication). However, the protein
138 concentration of serum is higher than that of VFM and the possibility/likelihood is therefore
139 that protein binding of tiamulin in serum did account for much of the increase of MIC.
140 The previously reported reduction in MICs of tulathromycin in 100% serum compared to
141 broth (Illambas *et al.*, 2009) might also be explained by differing pHs of the media, as frozen
142 calf serum had an increased pH (8.1 compared to 7.4) on thawing (Peter Lees - unpublished
143 data). This is in contrast to the present data, for which pH was higher in broth than in serum.
144 A previous report indicated that increasing the medium pH, from 7.3 to 8.0, increased
145 tiamulin's antimicrobial activity against *Staphylococcus aureus* by one dilution (Drews *et al.*,
146 1975). Godinho *et al.* (2005) also described a similar effect with tulathromycin and Godinho
147 (2008) reported that this effect seemed to be more important for fastidious species, such as *A.*
148 *pleuropneumoniae*. He suggested that this might be related to the increased buffering
149 capacity of serum in comparison with MHB / VFM. In the present study the pH values in
150 serum and VFM were 7.14 and 7.3, respectively, and both were less than the pKa of tiamulin
151 of 7.6. Moreover, the difference was less than 0.2 pH units, but nevertheless sufficient to
152 contribute to some of the observed differences in MIC between VFM and serum.

153 Tiamulin resembles other weak bases, such as tilmicosin and tulathromycin, in that plasma
154 concentrations bear little relation to therapeutic outcome in lung infections, except against
155 *Mycoplasma hyopneumoniae* (Burch, 2010). Concentrations of these weak bases in intra- and
156 extra-cellular lung compartments differ markedly (Mouton *et al*, 2008). Moreover, both
157 tilmicosin (Stoker *et al.*, 1996; Shryock and Scorneaux, 1997) and tulathromycin (Evans,
158 2005) have been reported to accumulate in leucocytes. Therefore, it has been proposed that
159 high tiamulin concentrations in leucocytes (Nielsen and Szancer, 1998), either polymorphs
160 and/or macrophages, could account for clinical outcome, especially as these cells engulf
161 bacteria to destroy them and they then become intracellular. Macrolides and pleuromutilins,
162 such as tylosin and tiamulin, respectively, have been shown to have activity against *Lawsonia*
163 *intracellularis* both in cell culture (Wattanaphansak *et al*, 2009) and tiamulin *in vivo*
164 (McOrist *et al*, 1996). On the other hand, the numbers of drug-loaded leucocytes or lung cells
165 and drug leakage following necrosis would be too small to account, alone, for the efficacy of
166 drugs of the macrolide and pleuromutilin groups. Other factors may be implicated. For
167 example, the immune modulating properties of these drugs may play a significant role in
168 contributing to clinical responses by reducing pneumonic lesions, for example by their anti-
169 inflammatory effects (Zhang *et al*, 2009), but these do not explain their antibacterial activity.
170 Currently, PK/PD integration using classical parameters of plasma concentration and MIC are
171 not possible for tiamulin and *A. pleuropneumoniae*.

172

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233
234

Table 1. Tiamulin MIC and MBC results against 20 isolates of *A. pleuropneumoniae* in Veterinary Fastidious Medium and 100% serum

APP isolate codes	Results in VFM		Results in 100% swine serum	
	MIC ($\mu\text{g/ml}$)	MBC ($\mu\text{g/ml}$)	MIC ($\mu\text{g/ml}$)	MBC ($\mu\text{g/ml}$)
Ref strain	12	12	NG	NG
DWC 15145	18	36	NG	NG
DWC 20759	16	18	NG	NG
DWC 10418	14	18	NG	NG
DWC 15140	12	14	NG	NG
DWC 10950	14	20	NG	NG
DWC 20758	10	10	NG	NG
DWC 10429	10	14	NG	NG
Ref strain	12	14	24	ND
DWC 6903	14	24	24	ND
DWC 10417	12	20	32	ND
DWC 10427	7	9	NG	ND
DWC 10953	12	32	NG	ND
DWC 18826	12	36	NG	ND
DWC 18829	12	18	NG	ND
Ref strain	12	14	24	ND
DWC 6908	12	24	NG	ND
DWC 10413	12	18	NG	ND
DWC 10423	12	14	NG	ND
DWC 12762	NG	NG	NG	ND

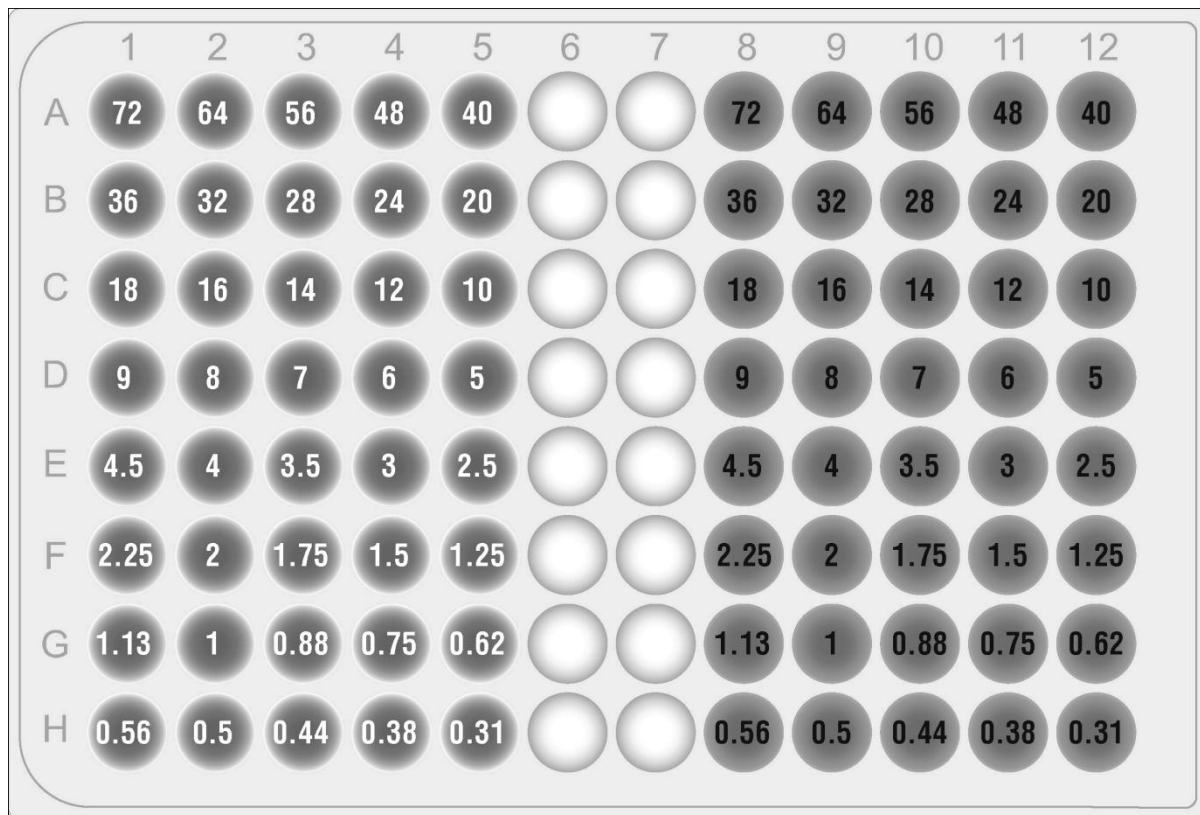
DWC 18792	10	24	NG	ND
DWC 18822	12	28	NG	ND
DWC 18824	12	36	24	ND

Key: APP = *A. pleuropneumoniae*; NG = No Growth; ND = Not Done

Table 2. MIC and MBC results of tiamulin against 20 isolates of *A. pleuropneumoniae* when grown in Veterinary Fastidious Medium

Results	MIC ($\mu\text{g/ml}$)	MBC ($\mu\text{g/ml}$)
MIC & MBC 50%	12	18
MIC & MBC 90%	14	36
MIC & MBC range	7-18	9-36
MIC & MBC mean	12.3	21.4
MIC/MBC ratio	1	1.74

Figure 1. Overlapping dilution series of tiamulin concentrations ($\mu\text{g/ml}$) employed for MIC/MBC determinations



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