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

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ABSTRACT

Tiamulin activity was measured against 19 UK field isolates of A. pleuropneumoniae collected between 2003 and 2009 and the type strain ATCC 27090 as a control, with the intention of comparing broth with serum as growth media. Broth microdilution MIC/MBC tests were performed in accordance with the Clinical and Laboratory Standards Institute (CLSI) guideline M31-A3, in ‘Veterinary Fastidious Medium’ (VFM) (supplemented Mueller-Hinton broth at pH 7.3) and in 100% swine serum. For improved precision, a modified, overlapping doubling-dilution series was used (tiamulin concentration range 0.3-72 µg/ml). The MBC was reported as the lowest concentration producing a 99.9% reduction in
bacterial density in the sub-cultured well contents, relative to the starting inoculum. The mean MBC/MIC ratio for tiamulin against *A. pleuropneumoniae* in VFM was low (1.74: 1), even though tiamulin is classed as a bacteriostatic drug. Only three of the 19 isolates and the reference strain grew in 100% serum and their MICs were higher than those determined in VFM. It is postulated that this difference was due to differences in pH of the matrices or binding of tiamulin to serum proteins or a combination of both factors.

**Keywords:**

*Actinobacillus pleuropneumoniae*  
PK/PD  
Tiamulin  
MIC  
MBC  
Serum culture

### 1. Introduction

Serum/plasma concentrations linked to indices such as MIC are well-established surrogates for most antimicrobial drugs. However, correlating the pharmacokinetics (PK) of tiamulin hydrogen fumarate concentrations in plasma with the pharmacodynamics (PD) measured as minimum inhibitory concentration (MIC) against *Actinobacillus pleuropneumoniae* has been unsuccessful. Using standardized Mueller-Hinton broth (MHB), MIC determinations based on the Clinical and Laboratory Standards Institute (CLSI) methods provided a susceptibility breakpoint ≤16 µg/ml against *A. pleuropneumoniae*. This value is much higher than the
plasma concentration achieved with therapeutic dose rates of tiamulin, suggesting that either
the MIC method of determination in artificial media does not reflect growth conditions in
biological fluids such as plasma or circulating concentrations of tiamulin differ significantly
from those at the site of infection. High intracellular concentrations of tiamulin in the lung
(Anderson et al., 1994; McKellar et al., 2004) and human polymorphonuclear leucocytes
(Nielsen and Szancer, 1998) were reported in comparison with plasma concentrations; both
tissues: plasma concentration ratios exceeding 18:1. In an artificial A. pleuropneumoniae
infection study in pigs, an isolate with a tiamulin MIC of 4µg/ml was effectively treated, both
clinically and bacteriologically, with 180 ppm tiamulin in the drinking water (Schultz et al,
1984; Burch and Klein, 2008). At this dosage, plasma concentrations of approximately
0.47µg/ml would be obtained, i.e. 1/8th of the MIC.
Studies using 2.5% serum in the culture medium (Godinho et al., 2005) and 100% serum
(Illambas et al., 2009) reduced the MIC values for tulathromycin against a variety of bacterial
respiratory pathogens of bovine origin but in particular Pasteurella multocida by 8 and 50
times, respectively, and against A. pleuropneumoniae (Godinho et al., 2005) by 32 fold. The
aims of this study were (1) to determine MICs, Minimum Bactericidal Concentrations
(MBCs) and MBC: MIC ratios for tiamulin against A. pleuropneumoniae using overlapping
sets of doubling dilutions to improve accuracy and (2) to determine whether the MIC and
MBC of tiamulin against A. pleuropneumoniae were reduced substantially by culturing the
organism in 100% swine serum in comparison with MHB.

2. Materials and methods
Tiamulin hydrogen fumarate (Denagard® - Novartis Animal Health Inc.) activity was
measured against 19 field isolates and one control type strain (ATCC 27090) of A.
pleuropneumoniae. All field strains were isolated during the period 2003-2009, from pigs
exhibiting clinical signs of pleuropneumonia, and were identified by veterinary diagnostic
laboratories. Following initial isolation, each strain was stored in a cryoprotective suspension
at -80°C and was subjected to no more than three subcultures from the original isolate. Strain
identification was confirmed before commencing the current study, on the basis of
morphological observations.

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

3. Results

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

For the 19 strains grown in VFM the MIC₅₀ and MIC₉₀ were 12 and 14 µg/ml, respectively,
and the MBC₅₀ and MBC₉₀ were 18 and 38 µg/ml, respectively. The MBC/MIC ratio for all
20 isolates was 1.74: 1 (Table 2). The pH of the serum was 7.14 and that of the VFM approximately 7.3 (range 7.29 - 7.33).

4. Discussion

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

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

The growth of *A. pleuropneumoniae* isolates in 100% serum was limited, only 4 of 19 strains showing visible growth. For these 4 strains the MICs were not reduced, as previously observed with 50% serum and 2.5 and 15% serum had no effect on MICs. In fact, MICs were twice as high in serum as in VFM in the present study. Conventionally, (e.g. CLSI standards),
MIC determinations are performed using a doubling dilution series of the antimicrobial agents. In that case, a doubling of the MIC is demonstrated by only a single dilution shift in the series and is thus regarded as being within the normal variability of the method. In the present study, we used a dilution series with narrower intervals and the MIC increase in serum is clearly genuine because it corresponds to a shift of 5 dilution steps in the series. Moreover, the lower MICs in VFM could be predicted from the higher pH of that medium, because the slightly more alkaline pH of VFM will lead to a higher unionised:ionised ratio of the drug. Another potentially significant factor is degree of protein binding of tiamulin, which was not measured in this study but has been reported at approximately 40% in swine serum at these concentrations (Ulrich Klein, personal communication). However, the protein concentration of serum is higher than that of VFM and the possibility/likelihood is therefore that protein binding of tiamulin in serum did account for much of the increase of MIC.

The previously reported reduction in MICs of tulathromycin in 100% serum compared to broth (Illambas et al., 2009) might also be explained by differing pHs of the media, as frozen calf serum had an increased pH (8.1 compared to 7.4) on thawing (Peter Lees - unpublished data). This is in contrast to the present data, for which pH was higher in broth than in serum. A previous report indicated that increasing the medium pH, from 7.3 to 8.0, increased tiamulin’s antimicrobial activity against Staphylococcus aureus by one dilution (Drews et al., 1975). Godinho et al. (2005) also described a similar effect with tulathromycin and Godinho (2008) reported that this effect seemed to be more important for fastidious species, such as A. pleuropneumoniae. He suggested that this might be related to the increased buffering capacity of serum in comparison with MHB / VFM. In the present study the pH values in serum and VFM were 7.14 and 7.3, respectively, and both were less than the pKa of tiamulin of 7.6. Moreover, the difference was less than 0.2 pH units, but nevertheless sufficient to contribute to some of the observed differences in MIC between VFM and serum.
Tiamulin resembles other weak bases, such as tilmicosin and tulathromycin, in that plasma concentrations bear little relation to therapeutic outcome in lung infections, except against *Mycoplasma hyopneumoniae* (Burch, 2010). Concentrations of these weak bases in intra- and extra-cellular lung compartments differ markedly (Mouton *et al.*, 2008). Moreover, both tilmicosin (Stoker *et al.*, 1996; Shryock and Scorneaux, 1997) and tulathromycin (Evans, 2005) have been reported to accumulate in leucocytes. Therefore, it has been proposed that high tiamulin concentrations in leucocytes (Nielsen and Szancer, 1998), either polymorphs and/or macrophages, could account for clinical outcome, especially as these cells engulf bacteria to destroy them and they then become intracellular. Macrolides and pleuromutilins, such as tylosin and tiamulin, respectively, have been shown to have activity against *Lawsonia intracellularis* both in cell culture (Wattanaphansak *et al.*, 2009) and tiamulin *in vivo* (McOrist *et al.*, 1996). On the other hand, the numbers of drug-loaded leucocytes or lung cells and drug leakage following necrosis would be too small to account, alone, for the efficacy of drugs of the macrolide and pleuromutilin groups. Other factors may be implicated. For example, the immune modulating properties of these drugs may play a significant role in contributing to clinical responses by reducing pneumonic lesions, for example by their anti-inflammatory effects (Zhang *et al.*, 2009), but these do not explain their antibacterial activity. Currently, PK/PD integration using classical parameters of plasma concentration and MIC are not possible for tiamulin and *A. pleuropneumoniae*.

5. References


Stoker, J., Parker, R., Spencer, Y., 1996. The concentration of tilmicosin in pig serum and respiratory tissue following oral administration of Pulomotil® via the feed at a level of 400g/tonne. Proc. International Pig Veterinary Society Congress, Bologna, Italy, 656.


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

<table>
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<th>Results in VFM</th>
<th>Results in 100% swine serum</th>
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<td>DWC 12762</td>
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Table 2. MIC and MBC results of tiamulin against 20 isolates of *A. pleuropneumoniae* when grown in Veterinary Fastidious Medium

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<tr>
<th>Results</th>
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<tr>
<td>MIC/MBC ratio</td>
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</table>

Key: APP = *A. pleuropneumoniae*; NG = No Growth; ND = Not Done
Figure 1. Overlapping dilution series of tiamulin concentrations (µg/ml) employed for MIC/MBC determinations

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