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1 [short communication for Veterinary Microbiology]

2

3 **Susceptibility testing of tulathromycin: interpretative breakpoints and**  
4 **susceptibility of field isolates.**

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13

14

15 **Abstract**

16 *In vitro* susceptibility tests were conducted on bovine and porcine respiratory  
17 pathogens isolated from European countries during 2004-2006 for susceptibility to  
18 tulathromycin using the recommended methodologies for broth microdilution. The  
19 results were compared with data from a similar survey conducted prior to launch in  
20 1998-2001 to monitor for any shift in susceptibility. The importance of maintaining  
21 the pH of the culture media within the range 7.2-7.4 was re-affirmed as a key factor in  
22 obtaining consistent minimum inhibitory concentration data. The use of recently  
23 established interpretative breakpoints would indicate that to date there has been no  
24 apparent decrease in susceptibility to tulathromycin since it became widely used  
25 across Europe.

26

27

28 **1. Introduction**

29

30 Following the introduction of tulathromycin for veterinary use in the treatment  
31 of respiratory disease in cattle and swine, a post-approval monitoring programme was  
32 established in order to monitor any changes in the susceptibility of field isolates of the  
33 key bacterial pathogens in countries where the product is approved. At the time of  
34 approval, minimum inhibitory concentration (MIC) ranges for control organisms used  
35 for quality control purposes had not been established, neither had interpretative  
36 standards been accepted for *in vitro* susceptibility testing with tulathromycin.  
37 Consequently it is thought probable that many laboratories have used the  
38 interpretative breakpoints established for tilmicosin, another macrolide antimicrobial  
39 used for the treatment of respiratory disease in cattle, and for which guidelines have  
40 been previously established by the Clinical and Laboratory Standards Institute (CLSI,  
41 2002). However, there are important structural differences between tilmicosin, which  
42 has a 16-membered ring, and tulathromycin, which has a 15-membered ring with  
43 three amine groups (Evans, 2005). These structural differences impart different  
44 pharmacokinetic/pharmacodynamic properties rendering the use of the same  
45 interpretative breakpoints inappropriate. Recently, breakpoints have been established  
46 for susceptibility testing of bovine respiratory pathogens to tulathromycin (Table 1)  
47 and these are expected to be incorporated into the next edition of the CLSI guidelines.  
48 Generally, the breakpoints for tulathromycin are one dilution higher than those for  
49 tilmicosin (CLSI, 2002). At this time the sponsor has developed similar guidance for  
50 porcine respiratory pathogens but these have yet to be accepted by the CLSI

51 veterinary sub-committee and should be regarded as provisional. In addition, initial  
52 investigation of the killing kinetics of tulathromycin indicated that susceptibility  
53 testing was subject to error arising from methodological variation in conditions (Reese  
54 et al., 2004; Evans, 2005). In order to ensure intra- and inter-laboratory consistency  
55 and repeatability of results, it is essential that a common methodology is employed by  
56 testing laboratories. This paper reports on a wide-ranging survey of the susceptibility  
57 to tulathromycin of bacterial isolates obtained from field outbreaks of bovine and  
58 porcine respiratory disease in Europe and reviews appropriate susceptibility testing  
59 methodology for tulathromycin.

60

61

## 62 **2. Materials and methods**

63

### 64 2.1 *Source of isolates*

65

66 Bacterial isolates were obtained from field studies conducted in various  
67 countries (Belgium, Czech Republic, Denmark, France, Germany, Italy, the  
68 Netherlands, Poland, Spain and the United Kingdom) as well as from library  
69 collections. All isolates were sourced from animals that had not received treatment  
70 with any antimicrobial. Species identification was performed using standard  
71 techniques. Prior to susceptibility testing all mycoplasmal isolates were stored at  
72 -70°C in vials containing 10% v/v glycerol and 1 mL Mycoplasma Medium™  
73 (Mycoplasma Experience Ltd, Reigate, Surrey, RH2 9BY, UK). All other bacterial  
74 isolates were stored on cryopreservative beads (Protect Beads™, Technical Services  
75 Consultants Ltd., Heywood, Lancashire, OL10 1DS, UK) at -75°C to -85°C.

76

77 2.2 *Determination of minimum inhibitory concentrations*

78

79 Susceptibility testing of the bacterial isolates to tulathromycin was performed  
80 in accordance with CLSI guidelines (CLSI, 2002) using a broth microdilution  
81 technique. For non-fastidious species the medium used was cation-adjusted Mueller-  
82 Hinton broth (CAMHB) with *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic  
83 acid (TES) buffer (Sensititre<sup>®</sup>, Trek Diagnostic Systems, Cleveland, OH, USA) in  
84 standard round-bottomed 96-well microdilution plates (Alpha Laboratories Sterile  
85 Microtitre plates, Hampshire, UK). Each well contained 50 µL and the dilution range  
86 for determination of MICs for tulathromycin was 0.06 to 64 µg/mL. Each plate  
87 contained three untreated wells for growth control purposes. MIC determinations for  
88 fastidious species (*Histophilus somni* and *Actinobacillus pleuropneumoniae*) were  
89 made using Veterinary Fastidious Medium (VFM) in a carbon dioxide enriched  
90 atmosphere (5% CO<sub>2</sub>). The test system was validated and routinely monitored by  
91 concurrently determining the MICs of *Escherichia coli* American Type Culture  
92 Collection (ATCC) 25922 (MIC 2-8 µg/mL), *H. somni* ATCC 700025 (MIC 4-32  
93 µg/mL), *A. pleuropneumoniae* ATCC 27090 (MIC 16-64 µg/mL) and *Staphylococcus*  
94 *aureus* ATCC 29213 (MIC 2-8 µg/mL) as quality control organisms. Immediately  
95 prior to each test the pH of the media was checked using a calibrated pH meter, to  
96 ensure it was within the pH range 7.2-7.4, and media found to be outside this range  
97 were discarded.

98 Bacterial isolates were retrieved from storage and sub-cultured onto Columbia  
99 sheep blood agar, incubated overnight at 35±1°C and checked visually for  
100 morphology and purity. Fastidious species, retrieved from storage, were sub-cultured

101 onto chocolate agar and incubated overnight at  $35\pm 1^\circ\text{C}$  in a carbon dioxide enriched  
102 atmosphere (5-7%  $\text{CO}_2$ ), prior to visual checking for morphology and purity. 0.5  
103 McFarland suspensions of test bacteria in saline were prepared by suspending 3-5  
104 bacterial colonies in 5 mL of physiological saline and the optical density adjusted to  
105 0.08-0.1 at 625 nm to give approximately  $5\times 10^5$  cfu/mL (as per CLSI guidelines).

106 An equilibrated stock solution of tulathromycin was prepared (Trek Diagnostic  
107 Systems, Cleveland, OH, USA) and used for all susceptibility testing. Standardised  
108 cell suspensions were inoculated into the test plates within 30 minutes of preparation  
109 to give a final inoculum in each well of approximately  $5\times 10^5$  cfu/mL. A number of  
110 randomly selected inocula were titrated for confirmation of the approximate levels of  
111 cfu/mL. The colony counts obtained ranged from  $4.3 \times 10^5$  to  $8.8 \times 10^5$  cfu/mL and  
112 were regarded as acceptable. Diluted inoculum was kept at  $-4^\circ\text{C}$  or on ice until  
113 microtitre plates were inoculated. Each well was inoculated with 50  $\mu\text{L}$  of bacterial  
114 suspension. Plates were sealed to prevent desiccation and incubated for 16-20 hours  
115 (20-24 hours for fastidious species) at  $35^\circ\text{C}$  to ensure adequate growth in the drug-  
116 free control wells. Tests with poor growth in the drug-free wells were invalid and  
117 were repeated.

118 Mycoplasma isolates (stored at  $-70^\circ\text{C}$ ) were used to initiate further cultures  
119 which were vortexed after a one in ten dilution, and then further diluted to  $10^{-5}$  and  
120 inoculated onto plates to allow estimation of the number of colony forming units (cfu)  
121 in the broth culture. Mycoplasma suspensions for MIC determination in the range  $10^3$ -  
122  $10^5$  cfu/mL were prepared in fresh media at pH 7.5. Titres were determined by adding  
123 0.1 mL of suspension to 0.1 mL volumes of medium, and inoculating the mixture onto  
124 agar media. After 7 days incubation to allow for development of all colonies present,  
125 the number of colonies were counted.

126 The susceptibility of mycoplasmal isolates was determined using a  
127 modification of the method described by Tanner and Wu (1992) using Cellstar® plates  
128 (Greiner) with a single reading to determine end-points. Susceptibility testing was  
129 carried out using pre-counted, actively growing cultures. Proprietary media  
130 (Mycoplasma Media™, Mycoplasma Experience Ltd, Reigate, Surrey, RH2 9BY,  
131 UK) were composed of a broth base supplemented with bovine or porcine serum and  
132 yeast extract, and contained phenol red as a pH indicator and ampicillin as a bacterial  
133 inhibitor. Dilutions of mycoplasma broth culture were prepared in fresh media, prior  
134 to incubation at 36±1°C. Stock drug solutions were prepared fresh as described above.  
135 Each microtitre plate contained wells with uninoculated medium, medium at pH 6.8  
136 (to provide an end-point control) and inoculated medium alone as challenge controls.  
137 Plates were sealed with Falcon® (Becton Dickinson, 1 Becton Drive, Franklin Lakes,  
138 New Jersey 07417, USA) sealing strips, prior to incubation at 36±1°C. Growth was  
139 apparent as a pH dependent colour change in wells where growth was evident, caused  
140 by a growth-induced pH shift in the medium (changing from red to orange/yellow for  
141 glucose fermenters). Plates were inspected at the start, middle and end of each day  
142 until a colour change in the drug-free challenge control well was equal to or exceeded  
143 that for the end-point control well (pH 6.8) at which time the tests were then read.  
144 When end-points were not achieved or if the mycoplasma inocula fell outside the  
145 range 10<sup>3</sup>-10<sup>5</sup> cfu/mL the tests were repeated. The test system was validated and  
146 routinely monitored by concurrently testing *M. bovis* reference strain NC10131 and  
147 *M. hyopneumoniae* reference strain NC10110 with each set of susceptibility tests.

148 The MIC for any particular isolate was defined as the lowest concentration of  
149 tulathromycin to completely inhibit visible growth. The MIC<sub>50</sub> and MIC<sub>90</sub> values were



150 defined as the lowest concentrations capable of inhibiting the growth of 50% and 90%  
151 of isolates, respectively.

152

### 153 3. Results

154

155 A total of 170 isolates were of bovine origin (*Mannheimia haemolytica* 33,  
156 *Pasteurella multocida* 44; *H. somni* 30; and 63 *M. bovis*) and 133 isolates were of  
157 porcine origin (*A. pleuropneumoniae* 53; *P. multocida* 53; and 27  
158 *M. hyopneumoniae*). The MIC results for each species are summarised in Table 1 and  
159 the interpretative breakpoints would indicate that at these MIC<sub>90</sub> values, the bacterial  
160 species tested were susceptible to tulathromycin. However, no adequate interpretation  
161 of breakpoints has, as yet, been determined for *M. bovis* or *M. hyopneumoniae*,  
162 although the low MIC data obtained for *M. hyopneumoniae* would suggest general  
163 susceptibility. The results for *M. bovis* would indicate an MIC<sub>90</sub> of >64 µg/mL, but  
164 this is not necessarily predictive of a lack of clinical efficacy. Data has been  
165 previously presented to demonstrate that under controlled conditions, tulathromycin  
166 was clinically efficacious in treatment of respiratory disease in calves inoculated with  
167 a strain of *M. bovis* known to have an MIC for tulathromycin of >64 µg/mL (Godinho  
168 et al., 2005b). The MIC<sub>90</sub> values (and MIC range) for tulathromycin across all  
169 countries for bovine isolates determined in the current survey conducted with isolates  
170 collected during 2004-2006 were compared with those determined in the period 1998-  
171 2001 (Godinho et al., 2005a,b; McKelvie et al., 2005; Nanjiani et al., 2005) prior to  
172 the European product launch (Table 2).

173

174 **4. Discussion**

175

176 This is the first report to include interpretation of the antimicrobial  
177 susceptibility of respiratory pathogens to tulathromycin following its introduction  
178 onto the veterinary market. These data would indicate that despite the usage of the  
179 antimicrobial, in cattle and pigs there has as yet been no shift in susceptibility  
180 amongst the key pathogenic target species. Nevertheless, it is important that the  
181 situation continues to be monitored regularly. In recently published work comparing  
182 the MICs of porcine isolates of *P. multocida* collected over a two year period for a  
183 battery of antimicrobials by Kaspar and others (2007), the MIC<sub>90</sub> for tulathromycin  
184 was 2 µg/mL in 2004-5 and 1 µg/mL in 2005-6. Using the proposed new  
185 interpretative guidelines, with the exception of one resistant isolate from 2004-5  
186 (MIC = 64 µg/mL), all other isolates (639 isolates in 2004-5 and 471 in 2005-6) from  
187 both years were susceptible to tulathromycin (MIC range 0.03-4 µg/mL) (Kaspar et  
188 al., 2007).

189 In the work presented here, the sampling methodology utilised was broadly in  
190 agreement with that suggested by Wallmann (2006) for the monitoring of  
191 antimicrobial resistance in pathogenic bacteria from farm animals. As with any survey  
192 of susceptibility data it is accepted that there are a small number of shortcomings in  
193 the methodology employed. It was not possible, for example, to identify isolates from  
194 premises known to have been exposed to tulathromycin in the intervening period  
195 between the two periods surveyed. Neither was it possible to obtain isolates from the  
196 same farms or indeed in many instances the same countries. However, bias was  
197 reduced by not using data solely from herds where the product was known to either  
198 have been used or not to have been used. The only stipulation was that the isolate had

199 to be collected prior to administration of any antimicrobials during the current  
200 outbreak of respiratory disease. Surveys of antimicrobial susceptibility based upon  
201 isolates received by laboratories from practitioners are likely to be biased if the data  
202 include isolates from samples collected from problem animals/herds that have not  
203 responded to treatment and such data automatically selects in favour of a reduced  
204 susceptibility. It is not unknown for data biased in this manner to be used to support  
205 arguments in favour of restricting the veterinary usage of antimicrobials, especially in  
206 food producing species. While antimicrobials can provide valuable benefits in terms  
207 of animal welfare and control of clinical disease, it is important in relation to the  
208 development of resistance to monitor the field susceptibility of key pathogens to  
209 specific antimicrobials particularly if their use is widespread.

210 In order to ensure comparability of results, it is also important that a common  
211 methodology is employed during the conduct of susceptibility testing. Previous  
212 reports have indicated that alterations in the pH and variability between batches of  
213 media during susceptibility tests for tulathromycin against fastidious species  
214 (*H. somni* and *A. pleuropneumoniae*), could result in MIC values that differed by up  
215 to 4-fold (Reese et al., 2004; Evans, 2005; Godinho et al., 2005a). In addition, it was  
216 found that the results could be influenced by the presence of serum, with the range of  
217 MIC and MIC<sub>90</sub> considerably reduced in the presence of serum by a factor of at least  
218 4-fold for the aerobic species and 6 to 10-fold for the fastidious species, which was  
219 thought to be related to the increased buffering capacity provided by the serum  
220 (Godinho et al., 2005a). At this time it is not known whether the presence of serum  
221 could also affect susceptibility testing of mycoplasma species. Similar changes in  
222 MIC due to reduction of pH have been reported for erythromycin, azithromycin and  
223 clarithromycin (Pruul and McDonald 1992; Edney et al., 1998). The recently

224 established CLSI methodology for control organisms and interpretative guidelines for  
225 bovine isolates and the suggested guidance for porcine pathogens should provide  
226 improved repeatability of susceptibility testing for tulathromycin.

227

228

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230

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235

236

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**Table 1. Distribution and frequency of minimum inhibitory concentrations (MICs) for tulathromycin against bovine and porcine respiratory pathogens isolated from Europe during 2004-2006.**

| MIC<br>( $\mu\text{g/mL}$ )   | Bovine isolates                         |  |                                    |                                   | Porcine isolates                                 |  |   |
|---|---|--|------------------------------------|-----------------------------------|--|--|---|
|   | <i>Mannheimia</i><br><i>haemolytica</i> | <i>Pasteurella</i><br><i>multocida</i> | <i>Histophilus</i><br><i>somni</i> | <i>Mycoplasma</i><br><i>bovis</i> | <i>Actinobacillus</i><br><i>pleuropneumoniae</i> | <i>Pasteurella</i><br><i>multocida</i> | <i>Mycoplasma</i><br><i>hyopneumoniae</i> |
| $\leq 0.004$  | -                                       | -                                      | -                                  | -                                 | -  | -                                      | 1 (4%)                                    |
| 0.008   | -                                       | -                                      | -                                  | -                                 | -  | -                                      | 5 (19%)                                   |
| 0.015   | -                                       | -                                      | -                                  | -                                 | -  | -                                      | 7 (26%)                                   |
| 0.03  | -                                       | -                                      | -                                  | -                                 | -  | -                                      | 6 (22%)                                   |
| 0.06  | -                                       | -                                      | -                                  | -                                 | -  | -                                      | 4 (15%)                                   |
| 0.125   | -                                       | -                                      | -                                  | 1 (2%)                            | -  | -                                      | 4 (15%)                                   |
| 0.25  | 2 (6%)                                  | 2 (5%)                                 | -                                  | 3 (5%)                            | -  | 2 (4%)                                 | -   |
| 0.5   | 2 (6%)                                  | 8 (41%)                                | -                                  | 11 (17%)                          | -  | 19 (36%)                               | -   |
| 1   | 13 (39%)                                | 20 (45%)                               | 1 (3%)                             | 8 (13%)                           | -  | 28 (53%)                               | -   |
| 2   | 15 (45%)                                | 4 (9%)                                 | 18 (60%)                           | 6 (10%)                           | -  | 4 (8%)                                 | -   |
| 4   | 1 (3%)                                  | -                                      | 11 (37%)                           | 2 (3%)                            | -  | -                                      | -   |
| 8   | -                                       | -                                      | -                                  | -                                 | 8 (15%)  | -                                      | -   |
| 16  | -                                       | -                                      | -                                  | 2 (3%)                            | 44 (83%)   | -                                      | -   |
| 32  | -                                       | -                                      | -                                  | -                                 | 1 (2%)   | -                                      | -   |
| 64  | -                                       | -                                      | -                                  | -                                 | -  | -                                      | -   |
| >64   | -                                       | -                                      | -                                  | 30 (48%)                          | -  | -                                      | -   |
| Total No.<br>of Isolates  | 33 <sup>a</sup>                         | 44                                     | 30                                 | 63                                | 53   | 53 <sup>a</sup>                        | 27  |
| <b>Interpretative breakpoints for broth microdilution (<math>\mu\text{g/mL}</math>)</b> |   |  |                                    |                                   |  |  |   |
| S   | $\leq 16^\dagger$                       | $\leq 16^\dagger$                      | $\leq 16^\dagger$                  | -                                 | $\leq 32^\ddagger$                               | $\leq 16^\ddagger$                     | -   |
| I   | $32^\dagger$                            | $32^\dagger$                           | $32^\dagger$                       | -                                 | -  | $32^\ddagger$                          | -   |
| R   | $\geq 64^\dagger$                       | $\geq 64^\dagger$                      | $\geq 64^\dagger$                  | -                                 | $\geq 64^\ddagger$                               | $\geq 64^\ddagger$                     | -   |

<sup>a</sup>Percentages may not add to 100 due to rounding. <sup>†</sup>Standards approved by the CLSI subcommittee on veterinary antimicrobial susceptibility testing 2006.

<sup>‡</sup>Recommended standards, not currently approved by the CLSI. S = Susceptible, I = Intermediate, R = Resistant.

**Table 2. Comparison of sensitivity results for tulathromycin determined for European bovine and porcine isolates collected during 1998-2001 with those collected during 2004-2006.**

|                   | Bovine isolates                        |                       |                              |                       |                                 |                       |                         |                       |
|-------------------|--|-----------------------|------------------------------|-----------------------|---------------------------------|-----------------------|-------------------------|-----------------------|
|                   | <i>Mannheimia haemolytica</i>          |                       | <i>Pasteurella multocida</i> |                       | <i>Histophilus somni</i>        |                       | <i>Mycoplasma bovis</i> |                       |
|                   | 2004-2006                              | Pre-2002 <sup>†</sup> | 2004-2006                    | Pre-2002 <sup>†</sup> | 2004-2006                       | Pre-2002 <sup>†</sup> | 2004-2006               | Pre-2002 <sup>‡</sup> |
|                   | <i>n</i> =33                           | <i>n</i> =79          | <i>n</i> =44                 | <i>n</i> =88          | <i>n</i> =30                    | <i>n</i> =53          | <i>n</i> =63            | <i>n</i> =53          |
| MIC <sub>50</sub> | 1 µg/mL                                | 1 µg/mL               | 1 µg/mL                      | 0.5 µg/mL             | 2 µg/mL                         | 2 µg/mL               | 4 µg/mL                 | 0.25 µg/mL            |
| MIC <sub>90</sub> | 2 µg/mL                                | 2 µg/mL               | 1 µg/mL                      | 1 µg/mL               | 4 µg/mL                         | 4 µg/mL               | >64 µg/mL               | >64 µg/mL             |
| MIC range         | 0.25 – 4 µg/mL                         | 0.25 – 4 µg/mL        | 0.25 – 2 µg/mL               | 0.25 - 2 µg/mL        | 1-4 µg/mL                       | 1-4 µg/mL             | 0.125 - >64 µg/mL       | ≤0.063 - >64 µg/mL    |
|                   | Porcine isolates                       |                       |                              |                       |                                 |                       |                         |                       |
|                   | <i>Actinobacillus pleuropneumoniae</i> |                       | <i>Pasteurella multocida</i> |                       | <i>Mycoplasma hyopneumoniae</i> |                       |                         |                       |
|                   | 2004-2006                              | Pre-2002 <sup>†</sup> | 2004-2006                    | Pre-2002 <sup>†</sup> | 2004-2006                       | Pre-2002 <sup>§</sup> |                         |                       |
|                   | <i>n</i> =53                           | <i>n</i> =54          | <i>n</i> =53                 | <i>n</i> =65          | <i>n</i> =27                    | <i>n</i> =58          |                         |                       |
| MIC <sub>50</sub> | 16 µg/mL                               | 8 µg/mL               | 1 µg/mL                      | 0.5 µg/mL             | 0.03 µg/mL                      | 0.05 µg/mL            |                         |                       |
| MIC <sub>90</sub> | 16 µg/mL                               | 16 µg/mL              | 1 µg/mL                      | 2 µg/mL               | 0.06 µg/mL                      | 0.05 µg/mL            |                         |                       |
| MIC range         | 8 – 32 µg/mL                           | 4 – 16 µg/mL          | 0.25 - 2 µg/mL               | 0.25 – 2 µg/mL        | ≤0.004 – 0.125 µg/mL            | 0.05 – 0.4 µg/mL      |                         |                       |

<sup>†</sup> Godinho et al., 2005a. <sup>‡</sup> Godinho et al., 2005b. <sup>§</sup> McKelvie et al., 2005; Nanjiani et al., 2005.