

# Analysis of the Mutant Selection Window and Killing of *Mycoplasma hyopneumoniae* for Doxycycline, Tylosin, Danofloxacin, Tiamulin, and Valnemulin

## Running title: Antimicrobial targeting of *Mycoplasma hyopneumoniae*

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20 Number of words = 2596

## 21 Number of tables = 3

22 Number of figures = 4

23 **Abstract**

24 *Mycoplasma hyopneumoniae* is the major pathogenic microorganism causing  
25 enzootic pneumonia in pigs. With increasing resistance of *M. hyopneumoniae* to  
26 conventional antibiotics, treatment is becoming complicated. Herein, we investigated  
27 the mutant selection window (MSW) of doxycycline, tylosin, danofloxacin, tiamulin,  
28 and valnemulin for treating *M. hyopneumoniae* strain (ATCC 25934) to determine the  
29 likelihood of promoting resistance with continued use of these antibiotics. Minimum  
30 inhibitory concentration (MIC) values against *M. hyopneumoniae* were determined for  
31 each antimicrobial agent and ranged from  $10^5$  colony-forming units (CFU)/mL to  $10^9$   
32 CFU/mL based on microdilution broth and agar dilution methods. The minimal  
33 concentration inhibiting colony formation by 99% (MIC<sub>99</sub>) and the mutant prevention  
34 concentration (MPC) were determined by the agar dilution method with three  
35 inoculum sizes. Antimicrobial killing was determined based on MIC<sub>99</sub> and MPC  
36 values for all five agents. MIC values ranged from 0.001 to 0.25  $\mu$ g/mL based on the  
37 microdilution broth method, and from 0.008 to 1.0  $\mu$ g/mL based on the agar dilution  
38 method. MPC values ranged from 0.0016 to 10.24  $\mu$ g/mL. MPC/MIC<sub>99</sub> values were  
39 ordered tylosin >doxycycline >danofloxacin >tiamulin >valnemulin. MPC  
40 achieved better bactericidal action than MIC<sub>99</sub>. Based on pharmacodynamic analyses,  
41 danofloxacin, tylosin, and doxycycline are more likely to select resistant mutants than  
42 tiamulin and valnemulin.

43 **Keywords:** *Mycoplasma hyopneumoniae*, mutant selection window, killing activity,  
44 antimicrobials, enzootic pneumonia, antibiotic resistance

45

46 **1. Introduction**

47 *Mycoplasma hyopneumoniae* is the primary pathogen causing enzootic  
48 pneumonia, an important chronic respiratory disease in pigs resulting in high  
49 morbidity, low feed conversion rate, and considerable economic losses in the  
50 swine breeding industry [1]. Additionally, the disease makes pigs more susceptible to  
51 infection by secondary bacterial pathogens such as *Pasteurella multocida* and  
52 *Actinobacillus pleuropneumoniae* [2]. There are several kinds of antimicrobial agents  
53 that exhibit *in vitro* activity against *M. hyopneumoniae*, such as pleuromutilins,  
54 fluoroquinolones, macrolides, and tetracyclines [3, 4]. However, widespread use of  
55 these agents has resulted in acquired resistance of *M. hyopneumoniae* to  
56 fluoroquinolones, lincosamides, and macrolides [5-7]. Thus, in order to reduce the  
57 risk of drug resistance, it is necessary to develop novel drugs. However, even if new  
58 drugs are discovered, re-evaluation of antimicrobial dosing is essential, and is the  
59 main method aimed at preventing the emergence and expansion of drug-resistant  
60 strains.

61

62 The mutant selection window (MSW) hypothesis postulates that, for each  
63 antimicrobial-pathogen combination, an antimicrobial concentration range exists in  
64 which selective amplification of single-step, drug-resistant mutants occurs [8]. The  
65 upper and lower boundaries of the MSW are the mutant prevention concentration  
66 (MPC) and the minimal concentration that inhibits colony formation by 99% (MIC<sub>99</sub>),

67 respectively. The MPC is the minimum concentration that inhibits colony formation  
68 of the least antibacterial drug-susceptible mutant subpopulation. Therefore, when  
69 antimicrobial concentrations fall within the range of the MSW, this tends to lead to  
70 the enrichment of drug-resistant bacteria. Keeping drug concentrations above the  
71 MPC is likely to restrict the emergence of resistance [9]. This hypothesis has been  
72 verified by *in vitro* and *in vivo* experiments [10-13].

73

74 Because *M. hyopneumoniae* lacks a cell wall and is small bacterium (0.4 – 1.2  
75  $\mu\text{m}$ ), culture isolation conditions *in vitro* are a technical challenge. In particular,  
76 quantification by the viable count method to determine colony-forming units (CFU) is  
77 arduous. Therefore, studies on the pharmacodynamics of *M. hyopneumoniae* are  
78 scarce. In the current study we determined for the first time the MPC and identified  
79 the MSW for doxycycline, tylosin, danofloxacin, tiamulin and valnemulin against *M.*  
80 *hyopneumoniae* *in vitro*. We also used time-kill tests to determine the relative  
81 antibacterial effects at the MIC<sub>99</sub> and the MPC. MSW and MPC are useful parameters  
82 for optimizing dosing regimens, reducing the emergence of resistant mutants, and  
83 analyzing treatment failure [14]. In addition, using the CFU counting method, changes  
84 in the amount of *M. hyopneumoniae* after antibiotic action can be determined.

85

## 86 **2. Materials and Methods**

87 *Mycoplasma hyopneumoniae* standard strain ATCC 25934 was obtained as a  
88 freeze-dried powder from the China Institute of Veterinary Drug Control (Beijing,

89 China) and stored at -80°C. Broth medium base, cysteine, and NADH were purchased  
90 from Qingdao Hope Biological Technology. Sterile swine serum was bought from  
91 Guangzhou Ruite Biological Technology. The initial pH of the medium was 7.7 ± 0.1,  
92 and 1% agar was added to solid media.

93 Doxycycline (85.8%), danofloxacin (100%), tiamulin (99%), tylosin (82.6%),  
94 and valnemulin (98.3%) were obtained from Guangdong Dahuanong Animal Health  
95 Products. These five antibacterial agents were dissolved in Milli-Q water and  
96 sterilized by filtration. A 1280 µg/mL fresh stock solution of each antibacterial agent  
97 was prepared for each experiment.

98

99 **2.1 Determination of minimum inhibitory concentration (MIC)**

100 MIC values were determined as described previously [15]. Briefly, MIC values  
101 were calculated for 10<sup>5</sup>, 10<sup>6</sup>, and 10<sup>7</sup> CFU/mL *M. hyopneumoniae* cultures in the  
102 exponential phase. A 100 µL sample of exponential phase culture was added to an  
103 equal volume of drug-containing medium culture in a 96-well plate. A growth control  
104 (inoculum without antimicrobials), sterility control (sterile broth at pH 7.8), and  
105 end-point control (blank medium at pH 6.8) were included. Plates were cultured at  
106 37°C with 5% CO<sub>2</sub> in an incubator after being sealed. When the color of the growth  
107 control was the same as the end-point control, the MIC was determined as the  
108 minimal concentration of antibacterial agent that resulted in no color change.

109

110 MIC values were determined by the agar dilution method as described previously

111 [16]. A 10  $\mu$ L sample of *M. hyopneumoniae* culture ( $10^5$ – $10^7$  CFU) was placed on the  
112 surface of a plate in which wells contained 1.25–20  $\mu$ g/mL danofloxacin, 2–32  $\mu$ g/mL  
113 tiamulin, 4–64  $\mu$ g/mL tylosin, 5–80  $\mu$ g/mL doxycycline, or 0.16–2.56  $\mu$ g/mL for  
114 two-fold agar dilution analysis. All plates were incubated for at least 8 days.  
115 Meanwhile, growth control plates without antimicrobials were set up for each test,  
116 and all experiments were repeated three times. The lowest concentration without *M.*  
117 *hyopneumoniae* growth on agar plates was taken as the MIC value. Each test was  
118 repeated three times.

119

120 **2.2 Measurement of  $MIC_{99}$  and mutant prevention concentration (MPC)**

121  $MIC_{99}$  values were measured as reported previously [17] with modifications.  
122  $MIC_{99}$  drug concentrations were based on linear decreasing dilutions of MIC values.  
123 The antibiotic concentration ranged from  $1 \times MIC$  to  $0.5 \times MIC$  in sequential 10%  
124 dilution decreases. The quantity of bacteria in the logarithmic growth phase reached  
125  $10^7$  CFU/mL. Three 10  $\mu$ L drops of each diluted suspension were inoculated onto agar  
126 plates and cultured for at least 8 days as described above. Colony numbers between  
127 30 and 300 were counted.

128

129 The MPC is defined as the lowest drug concentration that prevents bacterial  
130 colony formation from a culture containing  $\geq 10^9$  CFU/mL bacteria [18]. We  
131 attempted different centrifugal methods for enriching *M. hyopneumoniae*. Ultimately,  
132 an 800 mL stationary growth phase culture was transferred into 20 tubes (each 50 mL),

133 tubes were centrifuged ( $5000 \times g$  for 20 min), and each bacteria solution was  
134 resuspended in 1 mL fresh medium. All 20 enriched cultures were combined into two  
135 15 mL tubes, centrifuged ( $5000 \times g$  for 20 min), and resuspended in 1 mL fresh  
136 medium for counting. The final concentration of *M. hyopneumoniae* was  $8.8 \times 10^9$   
137 CFU/mL.

138

139 MPC values were measured by the agar method as described previously [19].  
140 Briefly, 200  $\mu$ L samples of each enriched culture were inoculated onto agar plates  
141 containing various concentrations of antibiotic (six parallel solid plates per antibiotic  
142 concentration). These plates were incubated at 37°C with 5% CO<sub>2</sub> in a humidified  
143 incubator for 8–10 days. The lowest antibiotic concentration that resulted in no  
144 colony formation was considered the primary MPC (MPC<sub>pr</sub>). After a 20% linear drug  
145 concentration decrease in MPC<sub>pr</sub>, the MPC was tested again, and recorded as the  
146 lowest drug concentration preventing bacterial growth. Each test was repeated three  
147 times.

148

149 **2.3 Time-kill tests**

150 *In vitro* time-killing assays were performed as described previously [20]. Briefly,  
151 MIC<sub>99</sub> and MPC values for all five agents were tested. After adding 3.5 mL blank  
152 medium and 0.1 mL drug solution (40 times the target concentration) to each  
153 penicillin bottle, 0.4 mL exponential *M. hyopneumoniae* suspension with an inoculum  
154 size between  $10^5$  CFU/mL and  $10^9$  CFU/mL was added. Cultures were incubated at

155 37°C with 5% CO<sub>2</sub> for 48 h. Aliquots of 100 µL were collected from each culture at 0,  
156 3, 6, 9, 12, 24, 36, and 48 h. The viable cell number was determined via 10-fold serial  
157 dilutions and plating 10 µL of each diluted culture on drug-free agar. Growth controls  
158 (*M. hyopneumoniae* cultures without drugs) and sterility controls (5 mL medium at  
159 pH 7.8) were also included. Plates were incubated for at least 8 days at 37°C with 5%  
160 CO<sub>2</sub> in a humidified incubator. Each test was repeated three times.

161

### 162 **3. Results**

#### 163 **3.1 MIC determination**

164 MICs of danofloxacin, tiamulin, tylosin, doxycycline, and valnemulin against *M.*  
165 *hyopneumoniae* determined by the microdilution and agar dilution methods are shown  
166 in **Figure 1**. Values determined using the solid MIC method were 8-fold higher  
167 (tylosin), 4-fold higher (danofloxacin, doxycycline, and valnemulin), and 2-fold  
168 higher (tiamulin) than those determined by the liquid method at an identical inoculum  
169 size of 10<sup>5</sup> CFU/mL. In addition, as the inoculum size used in these assays was  
170 increased, the MIC values also increased. *M. hyopneumoniae* displayed its greatest  
171 sensitivity to valnemulin and its least to doxycycline

172

#### 173 **3.2 Determination of MIC<sub>99</sub>, MPC, and selection index (SI)**

174 MIC<sub>99</sub>, MPC, and SI values are shown in **Table 1**. The SI, the ratio of MPC to  
175 MIC<sub>99</sub>, reflects the ability of an antibacterial agent to induce resistant mutants. MIC<sub>99</sub>  
176 values for all antibacterial agents ranged from 0.0122 to 0.343 µg/mL, and MPC

177 values ranged from 0.016 to 10.24  $\mu\text{g}/\text{mL}$ . SI values ranged from 1.31 to 10.24, and  
178 were ranked valnemulin <tiamulin <danofloxacin <doxycycline <tylosin (low to high).  
179 Thus, valnemulin displayed the lowest SI value, while tylosin exhibited the highest SI  
180 value.

181

182 **3.3 In vitro killing analysis**

183 Time-kill curves of compounds against *M. hyopneumoniae* were obtained using  
184 three different inoculum sizes. Reductions in *M. hyopneumoniae* count with different  
185 inoculum sizes for MIC<sub>99</sub> and MPC are listed in **Table 2 and Table 3**. Danofloxacin,  
186 tiamulin, tylosin, and valnemulin achieved bactericidal activity against 10<sup>5</sup> CFU/mL  
187 *M. hyopneumoniae* with MIC<sub>99</sub> dosage, while doxycycline achieved bacteriostatic  
188 activity only. Colony count reductions recorded at the 48h time point 3.63, 3.68, 3.75,  
189 and 3.61 log<sub>10</sub> CFU/mL for danofloxacin, tiamulin, tylosin, and valnemulin,  
190 respectively, but only 1.4 log<sub>10</sub> CFU/mL for doxycycline. All five compounds  
191 achieved bactericidal activity against 10<sup>5</sup> CFU/mL *M. hyopneumoniae* with MPC  
192 dosage (**Figure 2**).

193 At a greater inoculum size (10<sup>7</sup> CFU/mL), danofloxacin, tiamulin, tylosin and  
194 valnemulin were bactericidal at the MIC<sub>99</sub> while doxycycline was bacteriostatic only.  
195 Colony count reductions recorded at the 48h time point were 5.15 log<sub>10</sub> CFU/mL for  
196 danofloxacin, 5.09 log<sub>10</sub> CFU/mL for valnemulin, 3.51 log<sub>10</sub> CFU/mL for tiamulin,  
197 4.13 log<sub>10</sub> CFU/mL for tylosin, and 1.16 log<sub>10</sub> CFU/mL for doxycycline. All five  
198 compounds achieved bactericidal activity against 10<sup>7</sup> CFU/mL *M. hyopneumoniae*

199 with MPC dosage (**Figure 3**).

200 Danofloxacin and valnemulin both achieved bactericidal activity against  $10^9$   
201 CFU/mL of *M. hyopneumoniae* cells at the MIC<sub>99</sub> concentrations while the other three  
202 were bacteriostatic only. Colony count reductions recorded at 48 h were danofloxacin  
203  $5.61 \log_{10}$  CFU/mL, valnemulin  $4.81 \log_{10}$  CFU/mL, tiamulin  $2.23 \log_{10}$  CFU/mL,  
204 tylosin  $2.39 \log_{10}$  CFU/mL and doxycycline  $2.43 \log_{10}$  CFU/mL. All compounds  
205 achieved bactericidal activity against  $10^9$  CFU/mL of *M. hyopneumoniae* at the MPC  
206 concentrations. Overall, the rank order of antibacterial agents for colony count  
207 reduction was danofloxacin >valnemulin >tylosin >tiamulin >doxycycline (**Figure 4**).

208

209 **4. Discussion**

210 *M. hyopneumoniae* is a major respiratory disease-causing pathogen in modern  
211 intensive pig farming worldwide. Although vaccine-based immunization is an  
212 important preventive measure for enzootic pneumonia, treatment with antibacterial  
213 agents is known to accelerate disease recovery and reduce disease-related  
214 complications. However, strains resistant to enrofloxacin and tylosin have appeared  
215 among clinically isolated strains [6, 7]. In particular, many fluoroquinolones are  
216 important antibiotics for the treatment of human infections, and are more likely to  
217 lead to cross-resistance. Due to the difficulties associated with *in vitro* culturing and  
218 viability counting (CFU measurements) for *M. hyopneumoniae*, systematic *in vitro*  
219 pharmacodynamic evaluation of antibacterial agents against *M. hyopneumoniae* is  
220 scarce. Thus, in the present study, *in vitro* pharmacodynamic indices of several

221 representative antimicrobial agents against *M. hyopneumoniae* were determined, and  
222 killing curves were plotted. To the best of our knowledge, this is the first study to  
223 explore the risk of *M. hyopneumoniae* resistance using MPC and MSW parameters.

224 MIC values determined by the liquid method were similar to those measured in  
225 previous studies [4, 5]. Using both liquid and solid agar methods, MIC values  
226 increased with increasing inoculum size; values obtained with a large inoculum were  
227 two to four times higher than those obtained with small inoculum. It has been reported  
228 previously that MIC values increase with increasing bacterial load [21, 22]. Among  
229 the five antibiotics tested, danofloxacin and tylosin were more sensitive to inoculum  
230 size for MIC determination. In an earlier report [23], a larger inoculum of  
231 *Staphylococcus aureus* had a more significant effect on the antibacterial activity of  
232 nafcillin and vancomycin than a smaller inoculum. Therefore, in cases of  
233 high bacterial inoculation, more careful consideration is required when selecting the  
234 MIC reference value for the relevant experiment. In clinical treatment, the MIC value  
235 should be determined for different bacterial counts according to the severity of animal  
236 infection to establish a better treatment plan.

237 The MPC is defined as the concentration of antibacterial drug that prevents the  
238 growth of large quantities of resistant sub-populations. Because culturing of *M.*  
239 *hyopneumoniae* is difficult *in vitro*, determination of MPC values is challenging; after  
240 much effort, *M. hyopneumoniae* was cultured to a cell density of  $10^7$ – $10^8$  CFU/mL.  
241 We tried a variety of enrichment methods to generate quantities of bacteria sufficient  
242 for determining MPCs, and eventually managed to measure MPCs for all five

243 antibiotics. Relationships between antimicrobial exposure, MPC/MSW values and  
244 antimicrobial resistance selection have been explored previously. For example, in  
245 *Staphylococcus aureus*, cefquinome concentrations below the MIC<sub>99</sub>,  
246 intermediate between the MIC<sub>99</sub> and MPC, and above the MPC resulted in the  
247 selection of mutants that differed in terms of the proportion of resistant and  
248 susceptible bacteria [11]. When the concentration of levofloxacin fell within the  
249 MSW, the sensitivity of *S. aureus* decreased and mutant subpopulations emerged [24].  
250 For *M. hyopneumoniae*, the reported danofloxacin, tylosin, and doxycycline  
251 concentrations in pigs fell within the MSW completely after an intramuscular dose of  
252 2.5 or 10 mg/kg (body weight); after oral administration at a dose of 20 mg/kg, C<sub>max</sub>  
253 values for danofloxacin, tylosin, and doxycycline were 0.45 ± 0.09, 2.71 ± 1.09, and  
254 2.44 ± 0.51 µg/mL [25-27]. Correlative MIC<sub>99</sub> and MPC values were 0.101 and 1.0,  
255 0.34 and 10.24, and 0.343 and 4.0 µg/mL, respectively. The reported tiamulin and  
256 valnemulin concentrations were comfortably above the MSW values only after oral  
257 administration at a dose of 10 or 25 mg/kg [28, 29]. Consequently, by combining the  
258 *in vivo* pharmacokinetic parameters and the *in vitro* pharmacodynamic results, we  
259 speculate that danofloxacin, tylosin, and doxycycline are more likely to select  
260 resistant mutants than tiamulin and valnemulin. Continued use of first-line  
261 antibacterial agents against *M. hyopneumoniae* according to current dosing regimens  
262 may therefore promote drug resistance selection, and hence limit their long-term  
263 efficacy in the treatment of endemic pneumonia in pigs.  
264 We determined the bactericidal effects of danofloxacin, tylosin, doxycycline,

265 tiamulin, and valnemulin against *M. hyopneumoniae* at various bacterial densities and  
266 drug concentrations. At three different inoculation amounts, doxycycline  
267 displayed bacteriostatic activity at MIC<sub>99</sub> dosage and bactericidal action at MPC  
268 dosage. At the highest inoculation amount, tiamulin and valnemulin acted  
269 as bacteriostatic agents at MIC<sub>99</sub> dosage and as bactericidal agents at MPC dosage.  
270 Danofloxacin exhibited the fastest sterilization rate. Moreover, valnemulin was highly  
271 sensitive to *M. hyopneumoniae*, and exerted an obvious bactericidal effect. These  
272 results showed that when the concentration of antibiotic equaled or exceeded the MPC,  
273 *M. hyopneumoniae* was rapidly killed. Drug concentrations at the MPC also reduced  
274 the chances of bacteria re-growing during drug exposure. These results are similar to  
275 those of an earlier report [30]. In this previous study, the bactericidal effect at MIC  
276 was slow and incomplete. However, at MPC and maximum serum or tissue drug  
277 concentrations, killing was more pronounced than at MIC, and increased with  
278 increasing duration of drug exposure.

279 The main limitation of the present study was that the *in vitro* pharmacodynamic  
280 determination of *M. hyopneumoniae* was only carried for the standard strain, and  
281 clinical isolates should be assessed to confirm our findings. Nevertheless, the present  
282 work represents a meaningful pilot study in this area. A second limitation is that all  
283 experiments were performed under ideal conditions *in vitro*, without considering the  
284 complexity of factors *in vivo*. Thus, *in vivo* experiments are currently being explored.

285 In conclusion, the present study was the first to establish pharmacodynamic  
286 analyses of five antimicrobial agents against *M. hyopneumoniae*. And we determined

287 MPC and MSW parameters to explore the risk of *M. hyopneumoniae* resistance. The  
288 results showed that the bactericidal action of MPC was better than MIC<sub>99</sub>, and the  
289 antibacterial effects of these drugs against *M. hyopneumoniae* are significantly  
290 different. These pharmacodynamic results are meaningful in choosing antimicrobials  
291 for therapy. And danofloxacin, tylosin, and doxycycline are more likely to select  
292 resistant mutants than tiamulin and valnemulin.

293 **Acknowledgments**

294 This work was supported by the National Key Research and Development  
295 Program of China (grant numbers 2016YFD0501300 and 2016YFD0501310).

296

297 **Author Contributions**

298 Methodology, software, validation, formal analysis, data curation, manuscript  
299 preparation, manuscript reviewing and editing, visualization, and project  
300 administration were performed by ZH. ZH, CM, ZZ, and LZ contributed to  
301 investigations. Resources were provided by XG, QC, XS, and HD. Supervision was  
302 provided by HD, and funding was acquired by HD.

303

304 **Conflict of Interest Statement**

305 The authors declare that the research was conducted in the absence of any  
306 commercial or financial relationships that could be construed as a potential conflict of  
307 interest.

308

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411 on clinical isolates of *Mannheimia haemolytica*. American Journal of Veterinary  
412 Research. 2015;76(10):860-8.

413

415 **Figure legends**

416

417 Figure 1. Minimum inhibitory concentration (MIC) determination for danofloxacin,  
418 tiamulin, tylosin, doxycycline, and valnemulin against *M. hyopneumoniae* in artificial  
419 medium using liquid and solid agar methods with inoculum sizes of  $10^5$ ,  $10^6$ , and  $10^7$   
420 CFU/mL.

421

422 Figure 2. *M. hyopneumoniae* killing curves at the minimal concentration inhibiting  
423 colony formation by 99% (MIC<sub>99</sub>) and at the mutant prevention concentration (MPC)  
424 with an inoculum of  $10^5$  CFU/mL.

425

426 Figure 3. *M. hyopneumoniae* killing curves at the minimal concentration inhibiting  
427 colony formation by 99% (MIC<sub>99</sub>) and at the mutant prevention concentration (MPC)  
428 with an inoculum of  $10^7$  CFU/mL.

429

430 Figure 4. *M. hyopneumoniae* killing curves at the minimal concentration inhibiting  
431 colony formation by 99% (MIC<sub>99</sub>) and at the mutant prevention concentration (MPC)  
432 with an inoculum of  $10^9$  CFU/mL.

434 **Table 1. Comparison of MIC<sub>99</sub>, MIC, MPC, and SI values for five antimicrobial**  
435 **agents tested against *M. hyopneumoniae***

	MIC <sub>99</sub> (µg/mL)	MIC (µg/mL)	MPC (µg/mL)	SI
Danofloxacin	0.101	0.125	1.0	9.90
Tiamulin	0.144	0.16	1.024	7.11
Tylosin	0.34	0.40	10.24	30.12
Doxycycline	0.343	0.50	4.0	11.66
Valnemulin	0.0122	0.016	0.016	1.31

436 MIC<sub>99</sub>, minimal concentration inhibiting colony formation by 99%.

437 MIC, minimum inhibitory concentration.

438 MPC, mutant prevention concentration.

439 SI, selection index (the ratio of MPC to MIC<sub>99</sub>).

440

441 **Table 2. Reduction in *M. hyopneumoniae* growth for three different inoculum**  
442 **sizes based on measured MIC<sub>99</sub> concentrations**

Inoculum Size (CFU/mL)	Time (h)	Danofloxacin (log <sub>10</sub> CFU/mL)	Tiamulin (log <sub>10</sub> CFU/mL)	Tylosin (log <sub>10</sub> CFU/mL)	Doxycycline (log <sub>10</sub> CFU/mL)	Valnemulin (log <sub>10</sub> CFU/mL)
10 <sup>5</sup>	24	3.38	0.52	1.88	0.77	1.96
	48	3.63	3.68	3.75	1.40	3.61
10 <sup>7</sup>	24	3.74	1.01	0.56	1.38	2.27
	48	5.15	3.51	4.13	1.16	5.09
10 <sup>9</sup>	24	3.52	1.29	1.48	1.84	2.34
	48	5.61	2.23	2.39	2.43	4.81

443 *M. hyopneumoniae* cultures at cell densities of 10<sup>5</sup>, 10<sup>7</sup>, and 10<sup>9</sup> CFU/mL were  
444 exposed to agents at MIC<sub>99</sub> dosage, and colonies were counted on drug-free plates.

445 Log<sub>10</sub> CFU/mL reductions in *M. hyopneumoniae* count from 24 to 48 h are expressed  
446 as positive values, and data are presented as means of triplicate experiments.

447

448 **Table 3. Reduction in *M. hyopneumoniae* growth for three different inoculum**  
449 **sizes based on measured MPC concentrations**

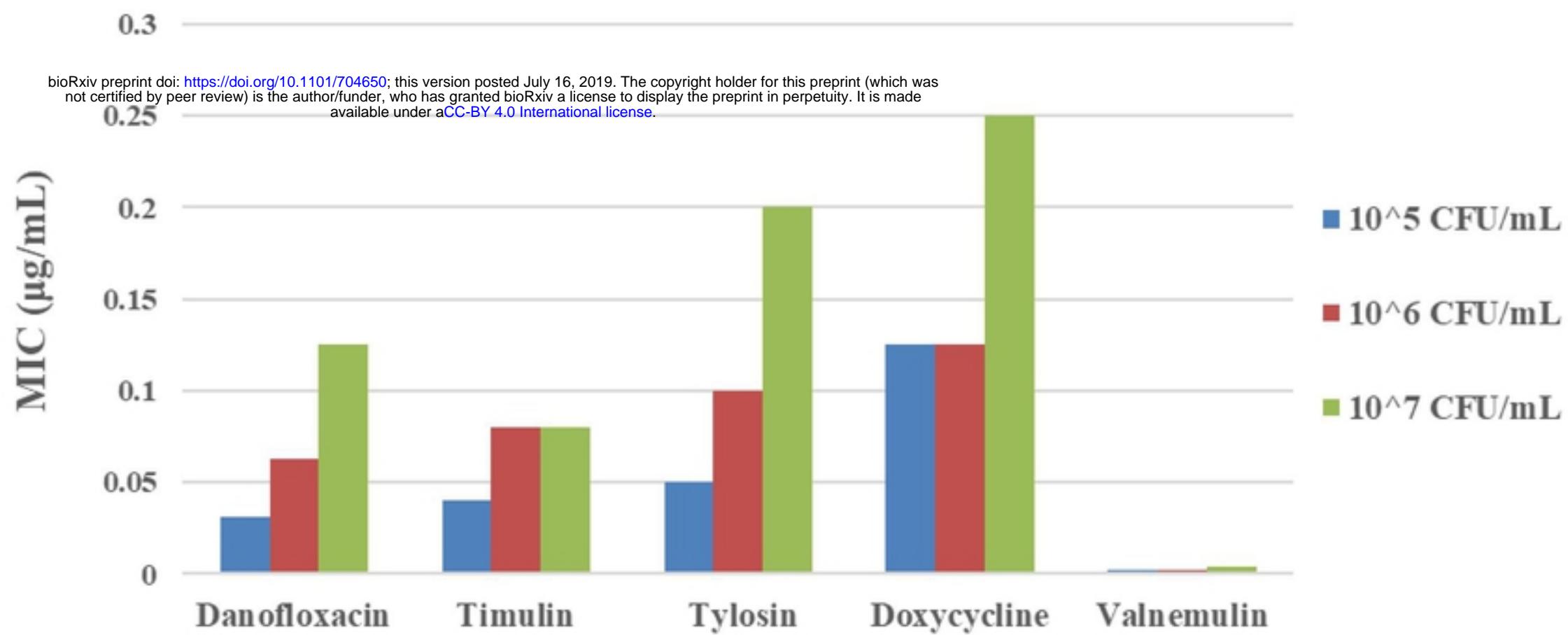
Inoculum size (CFU/mL)	Time (h)	Danofloxacin (log <sub>10</sub> CFU/mL)	Tiamulin (log <sub>10</sub> CFU/mL)	Tylosin (log <sub>10</sub> CFU/mL)	Doxycycline (log <sub>10</sub> CFU/mL)	Valnemulin (log <sub>10</sub> CFU/mL)
10 <sup>5</sup>	24	3.63	2.25	2.65	2.83	3.43
	48	3.63	3.68	3.75	3.72	3.61
10 <sup>7</sup>	24	3.08	3.16	2.47	2.57	2.99
	48	5.15	5.21	5.13	4.16	5.09
10 <sup>9</sup>	24	4.63	3.48	2.43	2.62	3.47
	48	7.03	4.13	4.36	3.96	4.90

450 *M. hyopneumoniae* cultures at cell densities of 10<sup>5</sup>, 10<sup>7</sup>, and 10<sup>9</sup> CFU/mL were  
451 exposed to agents at MIC<sub>99</sub> dosage, and colonies were counted on drug-free plates.

452 Log<sub>10</sub> CFU/mL reductions in *M. hyopneumoniae* count from 24 to 48 h are expressed  
453 as positive values, and data are presented as means of triplicate experiments.

454

## Liquid method



## Solid method

