

Sensitivity of Certain Porcine and Bovine Mycoplasmas to Antimicrobial Agents in a Liquid Medium Test Compared to a Disc Assay

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Friis, N.F. and J. Szancer: Sensitivity of certain porcine and bovine mycoplasmas to antimicrobial agents in a liquid medium test compared to a disc assay. Acta vet. scand. 1994, 35, 389-394. – The sensitivity of some porcine and bovine mycoplasmas to potent antimicrobial agents was examined. Minimal inhibitory concentration (MIC) values were estimated for *M. hyosynoviae*, *M. hyopneumoniae*, *M. dispar* and *M. bovis* against enrofloxacin, lincomycin, tetracycline, tiamulin and tylosin, in a liquid medium test and in a disc assay. All 6 examined strains of each species and the respective type strains were significantly inhibited. The greatest sensitivity was noted for tiamulin against strains of *M. hyosynoviae* with a final MIC₅₀ broth value of 0.025 µg ml⁻¹ and disc value of 0.03 µg per disc. Enrofloxacin was found very potent against *M. hyopneumoniae* with a final MIC₅₀ of 0.025 µg ml⁻¹ and 0.1 µg per disc, and for *M. dispar* with 0.05 µg ml⁻¹ and 0.03 µg per disc.

Most disc assay estimates in µg per disc were similar to or moderately greater than corresponding final broth figures in µg ml⁻¹. It may be possible to convert observed disc assay values into representative final broth MIC values for use in the clinic.

antibiotic; bovis; dispar; hyopneumoniae; hyosynoviae; lincomycin; quinolone; tetracycline; tiamulin; tylosin.

Introduction

In the search for effective antimicrobial therapy of mycoplasma infections in domesticated animals many agents have been examined, both in live animals (Hannan *et al.* 1982, Burch & Goodwin 1984, Ball & McCaughey 1985) and *in vitro*, usually in liquid medium (Williams 1978, Stipkovits *et al.* 1984, Cooper *et al.* 1993) but also using solid agar with the antimicrobial compounds incorporated (Hannan *et al.* 1989, ter Laak *et al.* 1991) or applied in dried paper discs (Wachowski & Kirchoff 1986).

From the various information it appears that many examined compounds possess a good suppressive effect on the replication of the mycoplasmas, especially in the *in vitro* studies,

although some variation in the minimal inhibitory concentration (MIC) values is apparent. These differences probably reflect minor diversities in the performance of the examinations. Notably higher MIC figures seem to result from work with the agents incorporated in dried paper discs placed on preseeded agar (Wachowski & Kirchoff 1986).

Among compounds for which very low *in vitro* MIC values have been reported are fluorquinolones, tiamulin and tylosin, but also lincomycin, macrolides and tetracyclines are often remarkably effective.

The aim of the present work has been to examine the inhibitory effect of some antimicrobial compounds on the *in vitro* growth of

pathogenic mycoplasmas from swine and cattle. In addition initial and final MIC values for agents dissolved in liquid medium have been determined and compared with the results obtained from disc diffusion tests, with the aim of providing a basis for routine antimicrobial sensitivity estimation.

Materials and methods

Design of the experiment

Antimicrobial agents were selected among the compounds usually recommended for treatment of infections with mycoplasmas in cattle and swine. The minimal inhibitory concentrations (MIC) were estimated as initial and final readings against the more important mycoplasma species cultivated both in liquid and on solid medium.

Strains of mycoplasma

The type strain and 6 Danish newer field isolates of each of 4 mycoplasma species were examined: the bovine *M. dispar*, type strain 462/2 and *M. bovis*, type strain PG45; the porcine *M. hyopneumoniae*, type strain "J" and *M. hyosynoviae*, type strain S16. The isolates originated from different farms (except 1 pig farm with 2 strains of *M. hyosynoviae*). The field strains were filtered through a 0.45 µm membrane and cloned once from a colony on solid medium and were used in 6th-10th passage. They were kept at -80°C.

Antimicrobial agents

The agents selected were the chemotherapeutic Enrofloxacin (a fluoroquinolone compound, Bayer Germany), and the antibiotics Lincomycin hydrochloride (Sigma L-6004), Tetracycline hydrochloride (Sigma T-7660), Tiamulin hydrogen fumarate (Leo Pharmaceutical Products, Ballerup, Denmark), and Tylosin tartrate (Elanco). The 4 antibiotics

were dissolved in distilled water and enrofloxacin in 5 mmol/l HCl at 40°C. They were added to liquid medium making serial approx. 2-fold dilutions in µg per ml, and to discs at approx. 3-fold dilutions in µg per disc, which were dried overnight at 37°C. Antibiotic Assay discs, 6.0 mm Whatman® were used.

Sensitivity experiment

A medium evolved for *M. hyopneumoniae* was used for this species and for *M. dispar*, and a Hayflick's type of medium was used for *M. bovis* and with added arginine/mucin for *M. hyosynoviae* (Friis *et al.* 1991). Solid medium was prepared from the former medium only. The liquid medium test was performed in tubes containing 1.8 ml of medium.

Mycoplasma stock cultures from -80°C were inoculated as 10-fold dilutions to 10⁻⁴ for each antimicrobial concentration and read visually for initial and final MIC values using tubes seeded with 10² to 10⁴ colour changing units. Initial reading was done after 2-4 days and final reading after 7-10 days when no progression had taken place over 2 days. Distinct colour shift (≥ pH 0.5, for *M. bovis* just 0.3-0.4) of the phenol red indicator in the medium was regarded as a positive reaction for growth, i.e. from yellow to red-bluish in cultures of *M. hyosynoviae* and from red to yellow in the 3 remaining species. Minimal inhibitory concentration (MIC) was determined as the lowest concentration indicating inhibition of growth as compared to controls without antimicrobials.

The solid medium test was performed using 60 mm petri dishes with just 1 antimicrobial-containing disc per plate and were incubated at 37°C in an atmosphere of 5-10 per cent CO₂ until growth. Plates with a non-confluent culture yielding numerous, but still distinct colonies were used. A zone from the edge of discs to the rim of normal colonies measuring at le-

Table 1. Antimicrobial sensitivity MIC values with porcine and bovine mycoplasmas against compounds in liquid medium and in dried discs.

	Tiamulin			Lincomycin			Tetracycline			Enrofloxacin			Tylosin		
	i	f	d	i	f	d	i	f	d	i	f	d	i	f	d
<i>M. hyosynoviae</i>															
Type strain	0.0025	0.025	0.03	0.10	0.50	0.3	0.10	0.25	3.0	0.25	0.5	1.0	0.025	0.050	0.03
125	0.0025	0.050	0.03	0.05	1.00	1.0	0.25	0.50	10.0	0.50	1.0	1.0	0.025	0.025	0.10
157	0.0025	0.025	0.10	0.10	1.00	1.0	0.25	1.00	3.0	0.50	1.0	1.0	0.025	0.050	0.10
561	0.0050	0.025	0.10	0.05	0.50	1.0	0.25	1.00	10.0	0.25	0.5	1.0	0.250	2.500	1.00
702	0.0025	0.025	0.03	0.10	0.50	1.0	1.00	2.50	10.0	0.50	1.0	1.0	0.500	2.500	0.30
712	0.0025	0.010	0.01	0.10	0.25	0.1	0.25	1.00	1.0	0.50	1.0	1.0	0.025	0.050	0.10
713	0.0025	0.025	0.01	0.05	0.25	0.3	0.25	1.00	10.0	0.50	1.0	1.0	0.025	0.050	0.10
<i>M. hypopneumoniae</i>															
Type strain	0.025	0.10	1.0	0.25	0.50	1.0	0.050	0.250	0.30	0.025	0.100	0.10	0.100	0.25	1.00
143	0.025	0.05	0.3	0.25	0.50	1.0	0.050	0.100	1.00	0.025	0.050	0.10	0.025	0.10	0.30
198	0.050	0.10	0.3	0.25	0.25	1.0	0.010	0.025	0.10	0.025	0.025	0.03	0.050	0.10	0.30
228	0.050	0.10	0.3	0.25	0.25	1.0	0.025	0.025	0.10	0.025	0.025	0.10	0.100	0.25	1.00
721	0.025	0.05	0.3	0.25	0.50	1.0	0.025	0.050	0.10	0.025	0.025	0.10	0.025	0.10	1.00
785	0.050	0.10	0.3	0.25	0.50	1.0	0.025	0.025	0.10	0.025	0.025	0.10	0.025	0.10	1.00
806	0.025	0.10	0.3	0.25	0.25	1.0	0.010	0.025	0.10	0.025	0.050	0.10	0.025	0.10	0.30
<i>M. dispar</i>															
Type strain	0.025	0.1	0.3	0.25	0.50	3.0	0.050	0.10	0.3	0.025	0.05	0.03	0.100	0.250	1.0
31	0.025	0.1	1.0	0.10	0.50	3.0	0.025	0.05	0.1	0.025	0.05	0.10	0.025	0.050	0.3
201	0.050	0.1	0.3	0.10	0.50	3.0	0.100	0.25	0.3	0.050	0.05	0.03	0.025	0.100	0.3
216	0.050	0.1	1.0	0.25	0.50	3.0	0.100	0.25	0.3	0.025	0.05	0.03	0.025	0.050	0.3
228	0.050	0.1	0.3	0.25	0.50	0.3	0.050	0.05	0.1	0.025	0.05	0.10	0.025	0.100	0.1
232	0.025	0.1	1.0	0.25	0.25	3.0	0.100	0.25	0.3	0.025	0.05	0.03	0.025	0.025	0.3
285	0.050	0.1	0.3	0.25	0.50	1.0	0.100	0.25	0.3	0.025	0.05	0.03	0.100	0.250	1.0
<i>M. bovis</i>															
Type strain	0.05	0.25	0.3	0.5	1.0	3.0	0.05	0.25	0.3	0.1	0.25	0.3	0.25	0.5	0.3
9	0.05	0.25	0.3	0.5	2.5	3.0	1.00	2.50	1.0	0.1	0.25	0.3	0.50	2.5	3.0
11	0.10	0.50	0.3	1.0	2.5	3.0	0.50	5.00	1.0	0.1	0.25	0.3	0.50	5.0	10.0
12	0.05	0.50	1.0	1.0	2.5	1.0	0.50	2.50	3.0	0.1	0.25	0.3	1.00	5.0	3.0
23	0.05	0.50	1.0	0.5	2.5	3.0	1.00	2.50	3.0	0.1	0.25	0.3	1.00	1.0	3.0
317	0.10	0.50	0.3	0.5	2.5	1.0	0.50	2.50	3.0	0.1	0.25	0.1	2.50	2.5	1.0
344	0.10	0.25	0.3	0.5	2.5	3.0	1.00	2.50	10.0	0.1	0.25	0.1	0.50	2.5	1.0

i, f = initial and final $\mu\text{g ml}^{-1}$ values; d = disc values in μg per disc.Table 2. MIC₅₀ values for mycoplasmas with agents in liquid medium and in discs.

	Tiamulin			Lincomycin			Tetracycline			Enrofloxacin			Tylosin		
	i	f	d	i	f	d	i	f	d	i	f	d	i	f	d
<i>M. hypopneumoniae</i> , type	0.025	0.1	1.0	0.25	0.5	1.0	0.05	0.25	0.3	0.025	0.1	0.1	0.1	0.25	1.0
6 field strains	0.025	0.1	0.3	0.25	0.25	1.0	0.025	0.025	0.1	0.025	0.025	0.1	0.025	0.1	0.3
<i>M. hyosynoviae</i> , type	0.0025	0.025	0.03	0.1	0.5	0.3	0.1	0.25	3.0	0.25	0.5	1.0	0.025	0.05	0.03
6 field strains	0.0025	0.025	0.03	0.05	0.5	1.0	0.25	1.0	10.0	0.5	1.0	1.0	0.025	0.05	0.1
<i>M. dispar</i> , type	0.025	0.1	0.3	0.25	0.5	3.0	0.05	0.1	0.3	0.025	0.05	0.03	0.1	0.25	1.0
6 field strains	0.05	0.1	0.3	0.25	0.5	3.0	0.1	0.25	0.3	0.025	0.05	0.03	0.025	0.05	0.3
<i>M. bovis</i> , type	0.05	0.25	0.3	0.5	1.0	3.0	0.05	0.25	0.3	0.1	0.25	0.3	0.25	0.5	0.3
6 field strains	0.05	0.5	0.3	0.5	2.5	3.0	0.5	2.5	3.0	0.1	0.25	0.3	0.5	2.5	3.0

i, f = initial and final $\mu\text{g ml}^{-1}$ values; d = disc values in μg per disc.

ast 5 mm with total or near-total inhibition was noted as sensitive. Reading was done after 3-5 days of incubation.

MIC₅₀ figures were calculated from individual readings as the smallest concentration able to inhibit at least 50% of the field strains of a single species.

Results

All 5 examined antimicrobial compounds (Table 1) were able to inhibit the replication of each of the 7 strains belonging to the 4 species in a concentration range of 0.0025 to 5.0 µg/ml. At the initial reading performed after 2-3 days in liquid medium, the MIC values for tiamulin was 0.0025 µg ml⁻¹ with strains of *M. hyosynoviae* and approx. 10-fold higher with strains of *M. hyopneumoniae* and *M. dispar*. Initial MIC for enrofloxacin and tylosin was found at 0.025 µg ml⁻¹ for many strains of *M. hyopneumoniae* and *M. dispar*. Also against *M. hyosynoviae* similar MIC values were noted for tylosin. With lincomycin initial MIC was usually 0.1 to 0.5 µg ml⁻¹. For *M. bovis* initial MIC values were found 5-10 times higher for all antimicrobials than for most other strains. Final MIC values in liquid medium were read after 7-10 days and in most cases found 2-5 times those of the initial readings.

The MIC values for compounds in dried paper discs placed on preseeded agar were expressed as µg per disc and as such were usually found similar to or up to 5 times higher than the final MIC liquid medium figures expressed as µg ml⁻¹.

MIC₅₀ concentrations expressing inhibition of at least 3 of the 6 field strains are given in Table 2 together with the values for the type strains. For *M. bovis* type strain, the initial, final and disc figures obtained with tetracycline and tylosin were up to 10 times lower than the field strain values.

Discussion

Some important antimicrobial agents with reported inhibitory effect on the replication of mycoplasmas were tested against a fairly low number of strains belonging to pathogenic bovine and porcine species (Cooper *et al.* 1993, ter Laak *et al.* 1991). All the examined compounds, enrofloxacin, lincomycin, tetracycline, tiamulin and tylosin showed a distinct effect on *M. hyosynoviae* and *M. hyopneumoniae* from swine, and *M. dispar* and *M. bovis* from cattle. As such the obtained results concord with earlier *in vitro* findings by others (Allan & Pirie 1981, Goodwin 1985, Hannan *et al.* 1989, Takahashi *et al.* 1978, Truscott & Ruhnke 1984).

The greatest potency noted throughout the present work was for tiamulin against *M. hyosynoviae*; a finding which corresponds to *in vitro* results obtained by Hannan *et al.* (1989) and also to experience from veterinary therapeutic work (Burch & Goodwin 1984, Madeiros 1984). The quinolone compound enrofloxacin was found remarkably effective against *M. hyopneumoniae* and *M. dispar* as was likewise found by Cooper *et al.* (1993) with another quinolone derivative, danofloxacin. Intraspecies strain variability in sensitivity was generally found moderate, especially for tiamulin and enrofloxacin. Such a feature may depend on little liability for development of resistance, but may also derive from limited use in therapy. For tylosin some strains among the examined species showed a reduced sensitivity. According to Cooper *et al.* (1993), this phenomenon may reflect an intensive medical use during many years.

The final MIC values as noted in liquid medium may be of special interest in the veterinary clinic, but the estimations are too laborious for routine work. As in the present work the final broth MIC₅₀ values in µg ml⁻¹ were found similar to or just moderately lower than

the disc MIC₅₀ values in µg/disc, it is suggested that the formula

$$Fb_c = \frac{Fb_{kn} \times Do}{D_{kn}}$$

be used to calculate the corresponding final broth MIC (Fb_c) from an observed disc method MIC (Do) by using the known final broth MIC₅₀ (Fb_{kn}) and disc MIC₅₀ (D_{kn}) values (Table 2). Finally, in another way, a corresponding MIC figure might be derived from a disc-dose-depending curve plotted in a system of co-ordinates.

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Sammendrag

Følsomhed for antimicrobielle stoffer hos visse svine- og kvægmykoplasmer undersøgt i flydende medium og sammenlignet til en disk metode.

Nogle vigtige svine- og kvægmykoplasmer blev undersøgt for følsomhed over for specielt udvalgte, potente antimikrobielle forbindelser. Den laveste hæmmende koncentration (MIC værdi) blev bestemt for 6 nye isolater samt typestammen af de porcine arter, *M. hyosynoviae* og *M. hyopneumoniae*, samt de bovine arter *M. dispar* og *M. bovis*, over for enrofloxacin, lincomycin, tetracyclin, tiamulin og tylosin. Der blev anvendt både en test i flydende medium med tidlig og slut registrering samt en disk metode.

Alle isolater samt typestammerne fandtes tydeligt følsomme under begge forsøgsbetingelser. Den største følsomhed blev registreret for tiamulin over

for *M. hyosynoviae*, hvor der fandtes en slut MIC₅₀ værdi på 0.025 µg pr. ml og en disk værdi på 0.03 µg pr. disk. Også enrofloxacin fandtes bemærkelsesværdigt aktivt over for *M. hyopneumoniae* med slut MIC₅₀ på 0.025 µg pr. ml og 0.1 µg pr. disk og over for *M. dispar* på 0.05 µg pr. ml og 0.03 µg pr. disk. I forsøg med enrofloxacin, lincomycin og tiamulin fandtes kun mindre stammevariation inden for mykoplasmaarterne, medens spredningen var noget

større for tetracyclin og tylosin; en forskel, som kunne afspejle intensiteten i brugen over årene.

I de fleste forsøg fandtes disk værdierne i µg pr. disk på niveau med eller moderat højere end de tilsvarende slutværdier i µg pr. ml. Det synes derfor muligt at konvertere observerede disk assay værdier til repræsentative slutværdier i µg pr. ml ved hjælp af en simpel omregningsfaktor med henblik på udnyttelse i klinikken.

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