ORIGINAL RESEARCH

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Testing antimicrobial susceptibility against *Mycoplasma hyopneumoniae* in vitro

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Summary

Objective: To determine and compare the susceptibility of 14 field isolates of *Mycoplasma hyopneumoniae* to 12 antimicrobial agents.

Methods: Isolates were cultured directly from porcine lung sections and positively identified as *M. hyopneumoniae* using an indirect-fluorescent antibody (IFA) method. A Sensititre® plate broth microdilution technique was used to determine the minimum inhibitory concentrations (MICs) of each of the antimicrobials. Inocula concentrations were determined by serial tenfold dilutions of the 14 *M. hyopneumoniae* isolates. An inoculum density of 10^3 CCU per mL was chosen as the target concentration of *M. hyopneumoniae*. Each well of a Sensititre® plate, prepared with the 12 antimicrobials, was inoculated with 50 µL of diluted culture and incubated at 37°C, then growth was monitored for 3–5 days. The MIC was recorded as the lowest concentration of each antimicrobial to inhibit visible color change.

Results: All of the isolates were resistant to the β-lactams ampicillin (MIC > 32 µg per mL) and ceftiofur (MIC > 8 µg per mL), and to the sulfonamide trimethoprim (MIC > 16 µg per mL)/sulfamethoxazole (MIC > 304 µg per mL). For the macrolides, >70% of the mycoplasma demonstrated MICs > 16 µg per mL for erythromycin and >70% were ≥ 64 µg per mL for tylosin; however, for tilmicosin (a newer semisynthetic macrolide) 93% of the isolates demonstrated MICs ≤ 8 µg per mL. Lincomycin MICs were ≤ 1 µg per mL for 93% of the isolates. All of the isolates demonstrated MICs ≤ 0.5 µg per mL for tetracycline and 93% were ≤ 4 µg per mL for enrofloxacin. Degree of susceptibility of *Mycoplasma* to the three members of the aminoglycoside group tested varied: 100% demonstrated MICs ≤ 2 µg per mL for gentamicin, 93% were ≤ 4 µg per mL for apramycin, and for spectinomycin 86% were at an MIC ≤ 4 µg per mL.

Implications: Based on the in vitro results obtained, further evaluation of tilmicosin and enrofloxacin for use against *Mycoplasma* in swine is warranted.

Keywords: swine, *Mycoplasma hyopneumoniae*, antimicrobial, susceptibility

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Mycoplasma hyopneumoniae is an endemic swine pathogen that causes economically significant pneumonia in commercial swine herds,[1,2] and can predispose swine to subsequent respiratory bacterial infection.[1,3] Vaccine use and previous *Mycoplasma* exposure have been demonstrated to protect swine from infection.[3,4] Antimicrobial efficacy studies with *M. hyopneumoniae*-infected swine have been few,[5,6] and only one antimicrobial is approved for use against *M. hyopneumoniae* in the United States (lincomycin).

As new claims for existing swine antimicrobials are considered and new agents are developed, it becomes necessary to have a standardized antimicrobial susceptibility test. The broth microdilution technique offers advantages in economy and convenience compared to agar and broth tube dilution.[7,8] With broth microdilution, diluted culture is dispensed into a 96-well plate that contains dilutions of the antimicrobial agents. More recently, commercially prepared Sensititre® plates have been used with the broth microdilution technique for testing *M. hyopneumoniae*.[9]

The present study used the Sensititre® plate broth microdilution technique to compare the susceptibility of antimicrobial agents against 14 field isolates of *M. hyopneumoniae*. Seven classes of antimicrobials were represented:

- β-lactams,
- sulfonamides,
- macrolides,
- lincosamides,
- tetracyclines,
- aminoglycosides, and
- quinolones.

In all, 12 antibiotics were tested (Table 1). Although many of these are

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used in swine to treat or control bacterial diseases, not all are approved by the United States Center for Veterinary Medicine (CVM) for treating *Mycoplasma* infection.

Materials and methods

Mycoplasma hyopneumoniae field isolates

Because there are no well-characterized quality control reference strains available for *Mycoplasma*, we obtained 14 *M. hyopneumoniae* field isolates from swine lung specimens submitted to the Animal Disease Diagnostic Laboratory at Purdue University. Isolates were cultured in modified Friis broth[10] directly from porcine lung sections and positively identified as *M. hopneumoniae* using an indirect-fluorescent assay (IFA) method.[11] Antiserum to *M. hyorhinis* was incorporated in Friis broth to prevent contaminating the culture with *M. hyorhinis*.

Titration of *Mycoplasma* inocula

To determine the concentrations of *M. hyopneumoniae* isolate to use in assessing the minimum inhibitory concentrations (MICs) of the antimicrobials, the 14 *M. hyopneumoniae* isolates were titrated in a sterile 96-well Sensititre® plate. Each *M. hyopneumoniae* isolate was aliquoted to the second well of the second row of the plate, and serially diluted tenfold into each succeeding well. The control wells (the top row of the plate) included modified Friis medium only. The wells were covered with an adhesive seal to prevent evaporation and incubated at 37°C (98.6°F) for 3–5 days. The growth of *Mycoplasma* isolates in the plates was monitored daily for a period of 3–5 days. The endpoint (color change unit [CCU] per mL) was read as the reciprocal of the dilution that had a stable color change (yellow) distinguishable from the next-lowest dilution.[12]

Determining minimum inhibitory concentrations

Ninety-six separate round-bottom well microtiter plates containing freeze-dried antimicrobials were prepared by Sensititre® (Sensititre Ltd., East Grinstead, Sussex, England). The antimicrobial agents were incorporated into the wells of the plate in a doubling dilution pattern over a range of concentrations appropriate to their potency (range 0.25–64 µg per mL, depending on the antibiotic) so that the full range of allowed dosage per manufacturer's recommendations would be represented in the test.

Each well of the Sensititre® plate was inoculated with 50 µL of *M. hyopneumoniae* culture. An inoculum density of 10^3 CCU per mL was chosen as the target concentration of *M. hyopneumoniae*. In those cases where the inoculum density varied from the target, the dilution closest to it was used (i.e., 10^2 or 10^4 CCU per mL) for the MIC. The Sensititre® plate was covered with a transparent, self-adhesive seal, firmly fixed to prevent evaporation. The test plates were incubated at 37°C (98.6°F) for 3 days.

The MIC was recorded as the lowest concentration of each antimicrobial to inhibit visible color change in a well. Each isolate was tested twice and repeated if the end points for any antibiotic differed by more than one dilution.

Results

All of the isolates had high MICs to ampicillin (MIC > 32 µg per mL) , ceftiofur (MIC > 8 µg per mL), and trimethoprim/sulfamethoxazole (MIC > 16/304 µg per mL) (Table 1).

For the macrolides,

- more than 70% of the mycoplasma demonstrated MICs > 16 µg per mL for erythromycin, and
- more than 70% were ≤ 64 µg per mL for tylosin.

Table 1

MICs of 12 antibiotics (µg/mL) versus *M. hyopneumoniae*

	Isolate	P232	P5429	P2057	13359-4	P2218	P4713	P5456	P7331	P10617-2	P11796-1/a	P5215-1	P12536	P11796-1/b	P1170
Antibiotic	**CCU/mL**	10^2	10^2	10^2	10^2	10^3	10^3	10^3	10^3	10^3	10^3	10^3	10^3	10^3	10^3
Enrofloxacin		1	0.5	1	0.06	2	2	>4	1	1	1	2	1	1	1
Ceftiofur		>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8
Ampicillin		>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
Tilmicosin		≤0.25	4	>32	8	8	1	1	0.5	0.5	≤0.25	0.5	2	≤0.25	≤0.25
Tylosin		≤0.5	>64	16	64	>64	>64	>64	>64	>64	16	64	>64	32	>64
Erythromycin		0.25	>16	>16	>16	>16	>16	>16	>16	>16	>16	≤0.25	>16	8	4
Lincomycin		≤0.25	1	>32	0.5	1	0.5	0.5	0.5	≤0.25	≤0.25	≤0.25	0.5	≤0.25	≤0.25
Spectinomycin		≤1	8	4	2	2	2	2	2	2	2	4	16	2	2
Apramycin		0.5	8	4	4	4	4	4	1	4	4	4	4	4	4
Gentamicin		≤0.5	2	1	≤0.5	1	1	1	≤0.5	1	2	≤0.5	1	1	1
Tetracycline		≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	0.5	≤0.5	≤0.5
Trimethoprim/ Sulfa		ND	>16/ >304	>16/ >304	>16/ >304	>16/ >304	>16/ >304	>16/ >304	>16/ >304	>16/ >304	>16/ >304	16/ 304	>16/ >304	>16/ >304	>16/ >304

MICs were determined as described in the text at inoculum concentrations of 10^2 or 10^3 CCU/mL.

For tilmicosin, however, 93% of the isolates demonstrated MICs ≤ 8 µg per mL. Lincomycin MICs were ≤ 0.5 µg per mL for 93% of the isolates. All of the isolates demonstrated MICs of ≤ 0.5 µg per mL for tetracycline and 93% were ≤ 4 µg per mL for enrofloxacin.

The susceptibility of *Mycoplasma* to the three members of the aminoglycoside group tested varied somewhat:

- 100% demonstrated MICs ≤ 2 µg per mL for gentamicin,
- 93% were ≤ 4 µg per mL for apramycin, and
- 86% were at an MIC ≤ 4 µg per mL for spectinomycin.

The MICs for the majority of the antimicrobials tested did not change when the concentration of inoculum was varied by one dilution (± 10^1 CCU per mL) for a given isolate (data not shown). Thus, including the MICs for those strains tested at 10^2 CCU per mL was considered to be equivalent to the MICs for the eight isolates tested at 10^3 CCU per mL.

Discussion

Mycoplasma susceptibility studies have historically been conducted on a small number of isolates because mycoplasma is fastidious in its growth requirements and difficult to isolate, and because the test is complex to perform. We used Sensititre® plates and a technique similar to that used previously for porcine *Mycoplasma*[9] to minimize the complexity of the test for future expanded studies. However, inoculum density and initial and final pH of the culture medium (i.e., color change interpretation) remained the most important sources of test variation in the results we observed. The inoculum density could only be determined retrospectively, unlike bacterial susceptibility testing which is standardized to a known turbidity before it is added to the test plate. Consequently, some degree of inoculum variation between isolates was observed but it did not appear to result in any substantial differences in MICs relative to the MICs determined at the target inoculum.

Although ceftiofur and other β-lactams that inhibit cell wall synthesis are used therapeutically in swine, they appear to have no efficacy against *Mycoplasma*. The isolates were refractory to ceftiofur and ampicillin. *Mycoplasma* is intrinsically resistant to ceftiofur and ampicillin because there are no cell wall targets for those agents (ceftiofur and ampicillin were included in the test system to serve only as negative controls). This is consistent with reports regarding the susceptibility of *M. hyopneumoniae* to β-lactam antibiotics.[12]

The lack of activity of trimethoprim/sulfamethoxazole was in contrast to that observed in previous studies[13] and might be attributed to the possible presence of thymidine in the medium, which circumvents the inhibition of the folate pathway.

The MICs to erythromycin were high for most isolates (>16 µg per mL), which is consistent with previous investigations.[9,12] However, in an earlier study using a different testing methodology, MIC values for erythromycin ranged from 2.5–20 µg per mL.[14] Similarly, MICs for tylosin were ≥ 16 µg per mL for 93% of the isolates. In contrast, previous studies showed the effectiveness of tylosin against *M. hyopneumoniae* isolates with MICs well below 1 µg per mL.[9,12,14–17] Koh, et al.,[18] observed isolates to exhibit "moderate" MICs to tylosin (MIC = 3.2 µg per mL). These investigators used tube dilution, agar dilution, and microdilution on either their own microtiter plates or Sensititre® plates. The median MICs to tilmicosin in the present study were consistent with those reported by Inamoto, et al. (0.5 versus 0.2 µg per mL).[17] The apparent discrepancy with Koh's investigation could be due to differences in pH in the test systems. Macrolides are known to be less active at low pH, but the macrolides tested vary in pH sensitivity because they have different pKa values. As the pH becomes more basic, nonionized macrolides should cross the cytoplasmic membrane (and bind to the ribosome) more readily than the completely ionized macrolide at acidic pH. Because various macrolides have different pKa values (for example, tylosin has a pKa of 7.1 and erythromycin 8.8), as the pH becomes more acidic, the ionized macrolide becomes less able to penetrate into the cell to reach its target, reducing inhibitory activity.[19] Variation in the media composition, CO_2 atmosphere, and the duration of incubation all contribute to variation in the pH, which affects MICs.

Variation was also noted within the macrolide class. Minimum inhibitory concentrations were lower for tilmicosin on 13 of 14 of the same isolates for which high MICs were observed for erythromycin and tylosin. Since tilmicosin has low pKa values (7.4 and 8.5), it may be able to penetrate the cells over a wider pH range to inhibit growth.

The present study demonstrated the effectiveness of lincomycin and was similar to data generated before.[12,13,17] As with tilmicosin, the MICs against lincomycin were lower for 13 of 14 of the same isolates that exhibited high MICs against erythromycin and tylosin. Because lincomycin is the only antibiotic approved for use against *M. hyopneumoniae* in the United States, it may serve as a positive control drug when conducting efficacy testing in vitro or in swine. Although Lincocin® (Pharmacia-Upjohn) is currently the only antimicrobial labeled for use against mycoplasmal pneumonia in the United States, other products, including Tylan 200® Injection (Elanco), are indicated for therapy against other *Mycoplasma* species.

Three aminoglycosides were evaluated in the present study. Gentamicin was effective against all 14 isolates. Earlier studies showed that gentamicin had MICs of >50 µg per mL[13] and 0.25 µg per mL[16] for *M. hyopneumoniae*. Differences in strains used, antimicrobial doses chosen, and methodology may account for the differences in observed MICs. The activity of apramycin (modal MIC of 4 µg per mL) in the current study was lower by one to two dilutions than that observed by Tanner, et al.,[9] (modal MIC of 8 µg per mL) and was higher by two dilutions than that observed with gentamicin (modal MIC 1 µg per mL). The modal MIC against spectinomycin was 2 µg per mL, which is consistent with the results of Ter Laak, et al., (1 µg per mL).[12]

The MICs we observed against quinolone enrofloxacin were generally higher (0.5–2 µg per mL, with one isolate having an MIC >4 µg per mL) against the isolates in this study compared to other studies, in which slightly lower MICs (≤ 0.3µg per mL) were observed.[7,12] Koh, et al.,[18] found that their isolates were "sensitive" to enrofloxacin.

Tetracycline MICs were ≤ 0.5 µg per mL for all of the isolates tested, which is consistent with other reports.[7,12]

Tiamulin was not tested in this study because the number of antibiotics that could be included in the commercially obtained Sensititre® panels was limited; however, it is used in swine against mycoplasmal pneumonia and has low MICs to *Mycoplasma*.[20,21]

Because only a few isolates were included in this trial, and because there is a lack of data regarding the in vivo efficacy and pharmacokinetics of the antimicrobials, it is inappropriate to attempt to categorize any of these isolates as either "susceptible" or "resistant" on the basis of the present study.

The present study provided preliminary data regarding what agents may be useful in preventing, controlling, or treating *Mycoplasma*-associated respiratory disease. The low MICs we observed against tilmicosin and enrofloxacin suggest that these agents may be useful as a swine medication. Their potential should be evaluated in an investigation of the pharmacokinetic profiles of these agents based on the dose, duration, and route of administration. Because *Mycoplasma* acts as a predisposing pathogen in enzootic pneumonia, an effective antibiotic would also need to control bacterial respiratory pathogens to demonstrate meaningful clinical efficacy.

Implications

- Due to the small numbers of isolates tested, the result of this study should be considered to be preliminary.
- MICs to tilmicosin and enrofloxacin were low; further investigation is warranted to test the efficacy of these agents in vivo.

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