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In Vitro Susceptibilities of *Mycoplasma hyopneumoniae* Field Isolates

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The in vitro susceptibilities of 21 *Mycoplasma hyopneumoniae* field isolates were determined using a broth microdilution technique. One isolate showed acquired resistance to lincomycin, tilmicosin, and tylosin, while five isolates were resistant to flumequine and enrofloxacin. Acquired resistance against these antimicrobials in *M. hyopneumoniae* field isolates was not reported previously.

Mycoplasma hyopneumoniae causes enzootic pneumonia, a chronic respiratory disease in pigs resulting in considerable economic losses. Although appropriate vaccines are available to reduce the consequences of infection, medication with antimicrobials in feed or water is still a common practice. The use of antimicrobials, however, results in the selection of resistant bacteria. The antimicrobial susceptibility of *M. hyopneumoniae* field isolates has rarely been determined, and limited numbers of strains have been considered in these studies (5, 7, 8, 14, 16), mainly due to the fact that *M. hyopneumoniae* is a fastidious and slowly growing microorganism, making it difficult to obtain large numbers of field strains. In this study, the antimicrobial susceptibility of recently isolated *M. hyopneumoniae* field strains

TABLE 1. Antibiotics used on the herd during 1 year before isolation of *M. hyopneumoniae*

Herd	Isolate	Antibiotic(s) used in[a]:		
		Suckling piglets	Nursery piglets	Growth-finishing pigs
A	1	ENRO[1]	ENRO[1]	OXY[1]
B	2	DANO[1]	COL[5]	None
C	3[b]	ENRO[1]	COL[5]	TYL[4] or PEN[1]
D	4	AMX[2]	AMX-COL[5]	OXY[3] or DOX[3]
E	5	AMX[2] or PEN[2]	TMP-SULF[3]	TYL[3] or DOX[3]
F	6	ENRO[1]	COL[4], APR[4], AMX[4], OXY[4], ENRO[1]	None
G	7[b]	PEN[2], ENRO[1], NEO[1]	LIN[1], OXY[6], TYL[6]	LIN[1], OXY[6], TYL[6]
H	8	AMX[2]	LIN[1], OXY[6]	LIN[1], OXY[6]
I	9	None	COL[3]	None
J	10	None	AMX[3]	None
K	11	None	COL[5]	None
L	12	AMX[1], ENRO[1], PEN[2]	AMX[1], APR[3], COL[3], DOX[4], ENRO[1], TMP-SULF[3]	CEF[1], TYL[1]
M	13[b]	ENRO[1], PEN-STR[1], CEF[1], AMX[1]	AMX[5], COL[5], ENRO[2], CEF[1], PEN-STR[1]	OXY[5]
N	14	None	None	TMP-SULF[6]
O	15	TMP-SULF[1], AMX[1]	OXY[1]	OXY[1]
P	16[b]	AMX[1], ENRO[1]	AMX[5], AMX[1]	DOX[5], FFN[1]
Q	17	CEF[1], ENRO[1]	COL[5], TMP-SULF[4]	None
R	18[c]	None	None	LIN[1]
S	19[b]	ENRO[1]	ENRO[1], FLUM[3]	ENRO[1], PEN[1], OXY[1]
T	20	None	None	DOX[4]
U	21	COL[1], AMX[2]	COL[5]	TYL-DOX[5]

[a] Suckling piglets, 0 to 4 weeks of age; nursery piglets, 4 to 10 weeks of age; growth-finishing pigs, 10 weeks until slaughter. Drug abbreviations: AMX, amoxicillin; APR, apramycin; CEF, cefquinome; COL, colistin; DANO, danofloxacin; DOX, doxycycline; ENRO, enrofloxacin; FFN, florfenicol; FLUM, flumequine; LIN, lincomycin; NEO, neomycin; OXY, oxytetracycline; PEN, penicillin; STR, streptomycin; SULF, sulfonamides; TMP, trimethoprim; TYL, tylosin. A superscript numeral with a drug abbreviation indicates the way that drug was administered as follows: 1, injected (some pigs, in case of disease); 2, injected (all pigs, routine); 3, in feed (routine); 4, in feed (in case of disease); 5, in water (routine); 6, in water (in case of disease).
[b] Isolate for which flumequine and enrofloxacin MICs were high.
[c] Isolate for which tylosin, tilmicosin, and lincomycin MICs were high.

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TABLE 2. Initial and final MIC_{50}s, MIC_{90}s, and MIC ranges of antimicrobials against Belgian *M. hyopneumoniae* field isolates

Antimicrobial[a]	Reading	Field isolates (n = 21)			Reference (J) strain
		MIC_{50}	MIC_{90}	MIC range	MIC range
LIN	Initial	≤0.06	≤0.06	≤0.06–>8	<0.06
	Final	≤0.06	0.12	≤0.06–>8	0.25
LIN/SPT	Initial	≤0.06/0.12	≤0.06/0.12	≤0.06/0.12–0.25/0.5	<0.06/0.12
	Final	≤0.06/0.12	0.12/0.25	≤0.06/0.12–0.25/0.5	0.12/0.25–0.25/0.5
SPT	Initial	0.25	0.5	≤0.12–0.5	0.5
	Final	0.5	1	≤0.12–1	1–2
OXY	Initial	0.12	1	0.03–2	0.12
	Final	0.5	2	0.12–>2	1
DOX	Initial	0.12	0.5	0.03–1	0.06–0.12
	Final	0.5	1	0.12–2	0.5–1
ENRO	Initial	0.03	0.5	0.015–>1	0.015–0.03
	Final	0.06	0.5	0.03–>1	0.06
FLUM	Initial	1	>16	0.25–>16	0.5–1
	Final	2	>16	0.5–>16	2
GEN	Initial	≤0.12	0.5	≤0.12–1	0.25–0.5
	Final	0.5	1	≤0.12–1	0.5–1
FFN	Initial	≤0.12	0.25	≤0.12–0.5	0.25
	Final	0.25	0.5	≤0.12–1	1
TIA	Initial	≤0.015	0.12	≤0.015–0.12	0.03
	Final	0.03	0.12	≤0.015–0.12	0.06
TIL	Initial	0.25	0.5	≤0.25–>16	0.25
	Final	0.5	0.5	≤0.25–>16	0.5
TYL	Initial	0.03	0.06	≤0.015–>1	≤0.015–0.03
	Final	0.06	0.12	≤0.015–>1	0.12

[a] All data are given in micrograms per milliliter. MICs for the reference strain (J strain) were determined three times, and the results are included in this table.
[b] LIN, lincomycin; SPT, spectinomycin; OXY, oxytetracycline; DOX, doxycycline; ENRO, enrofloxacin; FLUM, flumequine; GEN, gentamicin; FFN, florfenicol; TIA, tiamulin; TIL, tilmicosin; TYL, tylosin.

was determined, and the antibiotic use on the originating herds was monitored.

For MIC determination, 21 *M. hyopneumoniae* field strains, isolated between 2000 and 2002 from 21 different farrow-to-finish pig herds in Belgium, were used (18, 19). The isolation and cultivation of *M. hyopneumoniae* were optimized by using earlier reports (12). The antibiotics used on the herds at the three main production stages during the year before *M. hyopneumoniae* isolation are mentioned in Table 1.

The MIC determination was performed according to guidelines written by Hannan (6). Briefly, 96-well, round-bottom microtiter plates (Sensititre Ltd., East Grinstead, England) containing stabilized, freeze-dried antimicrobials (Table 2) were used. Three wells on each plate were left antimicrobial free as a positive growth control. Freshly thawed *M. hyopneumoniae* isolates with known titers were diluted in nonselective Friis medium until the number of organisms reached 10^4 color-changing units/ml. Fifty microliters of the diluted culture was transferred into each well of the Sensititre plates. The *M. hyopneumoniae* type strain, ATCC 25634 (J strain), was used as the control strain and tested three times in order to estimate the reproducibility of the procedure. The plates were sealed using an adhesive foil and incubated at 36 ± 1°C for 14 days and observed daily. Growth of *M. hyopneumoniae* organisms was observed when the color of the medium changed from red to yellow (phenol red indicator). The initial and final MICs were recorded. The initial MIC was defined as the lowest antibiotic concentration to show no change in color when the growth control turned yellow, and the final MIC was defined as the lowest antibiotic concentration to show no change in color at 14 days after inoculation (13).

In Table 2, the initial and final MICs at which 50 and 90% of the isolates tested were inhibited (MIC_{50} and MIC_{90}, respectively) and the MIC ranges are presented for the 21 *M. hyopneumoniae* field strains and the three replicates of the J strain. The values for these replicates were equal or differed from each other by only one doubling dilution, indicating good reproducibility of the test. The initial MICs for the J strain were in agreement with values reported previously (7, 8, 14, 16).

A bimodal frequency distribution of MICs of the macrolides tylosin and tilmicosin as well as for the lincosamide antibiotic lincomycin was seen. The MICs of these antibiotics were clearly higher for one isolate, indicating acquired resistance. The MIC of tylosin for this isolate was also higher than the suggested breakpoint (7). Macrolides and lincosamides are chemically distinct but have similar modes of action and overlapping binding sites on the 23S rRNA of the 50S subunit of the bacterial ribosome. They act by blocking protein synthesis on assembled and functioning 50S ribosomal subunits (20). Acquired resistance against these antibiotics has not been described before for *M. hyopneumoniae* and has not been reported often for other mycoplasmas, most probably due to a limited number of strains having been tested. Only two *M. pneumoniae* strains, one *Ureaplasma urealyticum* strain, and two resistant *Mycoplasma hominis* isolates were isolated from humans (2). In animal mycoplasmas, acquired resistance to tylosin has been described for *Mycoplasma gallisepticum* (11), *Mycoplasma hyosynoviae* (1, 10), *Mycoplasma hyorhinis* (9, 10), and *Mycoplasma bovis* (17). No coresistance to lincomycin was observed for the tylosin-resistant *M. hyosynoviae* isolates. Lincomycin was not evaluated by Levisohn (11), and although tylosin- and lincomycin-resistant *M. bovis* strains were found by

Thomas et al. (17), the existence of coresistance was not reported. ter Laak et al. (15) found lincomycin-resistant *M. bovis* isolates, but those were susceptible to tylosin. The use of lincomycin in growth-finishing pigs from the herd where our macrolide-lincosamide-resistant *M. hyopneumoniae* strain was isolated may have contributed to the selection of antibiotic resistance.

For five isolates, the MIC of flumequine was >16 μg/ml, which is higher than the previously proposed breakpoint of ≥16 μg/ml (7). For these isolates, the MIC of enrofloxacin was ≥0.5 μg/ml, while the MIC_{50} was 0.06 μg/ml. This rather high frequency of acquired resistance against fluoroquinolones is unusual. Although fluoroquinolone-resistant *M. hominis* isolates have been reported (3), resistance against these antibiotics in human-associated mycoplasmas has rarely been reported. Hannan et al. (7) described acquired resistance against flumequine in avian, porcine (but not *M. hyopneumoniae*), bovine, ovine, and caprine mycoplasmas, and Thomas et al. (17) isolated enrofloxacin-resistant strains from bovines. A possible explanation for the high prevalence of fluoroquinolone resistance in the present study might be the frequent use of enrofloxacin to treat *Escherichia coli* diarrhea in suckling and nursery piglets. The MIC of enrofloxacin for *M. hyopneumoniae* isolate 19 was >1 μg/ml. In the originating herd, fluoroquinolones were used in suckling, nursery, and growth-finishing pigs.

The MICs of oxytetracycline and doxycycline did not show a clear bimodal frequency distribution range (4), and the MICs of oxytetracycline were lower than the suggested breakpoint (7), indicating no acquired resistance against these antibiotics; nevertheless, in 62% of the herds selected for this study tetracycline antibiotics were used to treat nursery and growth-finishing pigs.

In conclusion, this study is the first description of acquired resistance in *M. hyopneumoniae* field isolates to macrolides, lincosamides, and fluoroquinolones. Resistance against other antimicrobials was not detected, confirming that antimicrobial resistance does not yet pose a major problem for the treatment of *M. hyopneumoniae* infections (7, 8, 16). However, the rather high frequency of fluoroquinolone resistance is worrying and warrants prudent use of these antibiotics.

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