***In Vitro* Susceptibility of *Mycoplasma hyosynoviae* and *M. hyorhinis* to Antimicrobial Agents**

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ABSTRACT. Fifty-four Japanese strains of *Mycoplasma hyosynoviae* isolated from porkers during 1980 to 1995, and 107 Japanese strains of *M. hyorhinis* isolated from piglets with respiratory disease during 1991 to 1994 were investigated for the *in vitro* activities of 13 antimicrobial agents [josamycin, tylosin, spiramycin, kitasamycin, erythromycin, lincomycin (LCM), kanamycin (KM), chloramphenicol (CP), thiamphenicol (TP), tiamulin (TML), oxytetracycline (OTC), chlortetracycline (CTC), and enrofloxacin (ERFX)] by the agar dilution method. Of the drugs tested TML showed the highest activity with minimum inhibitory concentration (MIC) of 0.013 to 0.1 µg/ml (MIC₉₀; 0.05 µg/ml) against strains of *M. hyosynoviae*, and 0.2 to 0.78 µg/ml (MIC₉₀; 0.39 µg/ml) against strains of *M. hyorhinis*. ERFX, LCM, most of the 16-membered macrolide antibiotics and tetracyclines also showed low MICs against both mycoplasma species. The susceptibility of KM, CP and TP to the mycoplasmas was considered to be of a secondary grade. Two of 54 strains of *M. hyosynoviae*, and 11 of 107 strains of *M. hyorhinis* showed resistance to all 14- and 16-membered macrolide antibiotics tested. Tetracyclines (OTC and CTC) showed a relatively broad MIC distribution from 0.1 to 6.25 µg/ml against the *M. hyosynoviae* strains tested. All of the strains isolated during 1980 to 1984 were susceptible at the concentration of 0.78 µg/ml or less (MIC₉₀; 0.78 µg/ml) to OTC and 1.56 µg/ml or less (MIC₉₀; 1.56 µg/ml) to CTC, while the susceptibility of strains isolated recently, during 1994 to 1995, was more than 0.78 µg/ml (MIC₉₀; 3.13 µg/ml) to OTC, and more than 1.56 µg/ml (MIC₉₀; 6.25 µg/ml) to CTC. — **KEY WORDS:** antibiotic, *Mycoplasma hyorhinis*, *Mycoplasma hyosynoviae*, resistance, susceptibility.

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Mycoplasmal arthritis, pneumonia, and polyserositis of pigs caused by *Mycoplasma hyosynoviae* or *M. hyorhinis* are very important diseases in the swine industry, as they cause retarded growth and reduced feed efficiency [17]. Though the best way to eradicate the disease is to replace the infected herd with a mycoplasma-free herd, this is impractical in Japan because of its high cost. No vaccines are available to control these diseases. Thus, at present, chemotherapeutic control is the most practical way to minimize the economic loss induced by mycoplasma infections. There are, however, few reports available on the *in vitro* effect of antimicrobial agents against several strains of *M. hyorhinis* and *M. hyosynoviae* in other countries [8, 18], and there is only one report on macrolide antibiotics (macrolides) inducible resistant strains of *M. hyorhinis* isolated from Japanese piglets [6]. This lack of information may be due in part to the fact that the isolation of these organisms still remains a difficult problem.

The present study was designed to investigate the *in vitro* activity of 13 antimicrobial agents which are commonly available for veterinary use against 54 Japanese isolates of *M. hyosynoviae* collected during 1980 to 1995, and to compare the susceptibility of the strains isolated in different years to the drugs examined. Furthermore, 107 recently collected (during 1991 to 1994) Japanese isolates of *M.*

hyorhinis were also investigated using the same drugs.

MATERIALS AND METHODS

Mycoplasmal strains: Fifty-four field-isolated strains of *M. hyosynoviae*, 107 field-isolated strains of *M. hyorhinis*, and the type strains, S16 (*M. hyosynoviae*) and BTS7 (*M. hyorhinis*), were used. The 54 strains of *M. hyosynoviae* were isolated from either the lung or the synovia of 54 porkers. Of the 54 field strains, 17 were isolated from lungs and 10 were isolated from arthritis, during 1980 to 1984. Of the remaining 27 field strains, 12 were isolated from lungs of 12 porkers (8 farms) and 15 were isolated from arthritis of 15 porkers (7 farms), during 1994 to 1995, in 3 slaughter houses in Japan. The 107 strains of *M. hyorhinis* were isolated from 38 piglets (1- to 3-month-old) with either respiratory disease or polyserositis from 20 farms in various parts of Japan, during 1991 to 1994. These strains were identified by the metabolism inhibition test [13]. The type strains were kindly supplied by R. Ross, Iowa state University.

Culture and culture condition: Primary isolation and preincubation of the organisms were carried out with slightly modified M broth and M agar media [6]. Briefly, The M broth consisted of PPLO broth (Difco), 21 g; bacteriological mucin (Difco), 5.0 g; heat-inactivated horse serum, 150 ml; 25% (wt/wt) extract of baker's yeast (Nitten Co., Ltd., Tokyo), 30 ml; 2.5% (wt/vol) thallium acetate, 10 ml; penicillin G potassium, 1 million IU; and distilled water, 810 ml. To prepare M agar, 1.2% (wt/vol) noble agar

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(Difco) was added to M broth. The inoculum in the broth medium was incubated at 37°C and that of the agar medium was incubated at 37°C with 5% CO₂ gas.

Antimicrobial agents: The following 13 antimicrobial agents which are approved for therapeutic use in industrial animals in Japan were used: josamycin (JM), tylosin (TS), spiramycin (SPM), kitasamycin (KT), erythromycin (EM), lincomycin (LCM), kanamycin (KM), chloramphenicol (CP), thiamphenicol (TP), tiamulin (TML), oxytetracycline (OTC), chlortetracycline (CTC), and enrofloxacin (ERFX). KT was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan, and the other drugs were supplied by the National Veterinary Assay Laboratory, Tokyo, Japan.

Test procedures: Serial twofold dilutions of the drugs were made in test tubes with M broth. One milliliter of each dilution was mixed with 9 ml of M agar. M agar plates without antimicrobial agents served as controls. Aliquots of the organisms (5 µl, containing approximately 10⁶ CFU/ml) were taken from the logarithmic phase and incubated onto the plates with a microplanter (Sakuma Co., Ltd., Tokyo, Japan). The plates were inoculated at 37°C with 5% CO₂ gas. MICs were read 4 days after inoculation. The lowest concentration of a drug inhibiting growth was defined as the MIC of the drug. Dwarf colonies that could not be observed by the naked eye were also regarded as a negative result.

RESULTS

***M. hyosynoviae*:** The distribution of the MICs obtained from the 55 strains tested are given in Table 1. TML showed the lowest MICs (highest activity) of 0.013 to 0.1 µg/ml (MIC₉₀; 0.05 µg/ml). Among the 16-membered macrolides, JM, TS, and KT had higher activities (0.05 to 0.78 µg/ml, MIC₉₀; 0.39 µg/ml, respectively) than SPM (3.13 to 6.25 µg/ml, MIC₉₀; 3.13 µg/ml) against all the strains tested except two strains (IS-1 and IS-2). Among the 14-membered macrolides, EM had the lowest activity against the strains of *M. hyosynoviae* (MIC₉₀; >100 µg/ml). LCM and ERFX had similar levels of activity as the 16-membered macrolides (0.2 to 0.39 µg/ml, MIC₉₀; 0.39 µg/ml, respectively). Whereas the MICs of tetracyclines (OTC and CTC) were distributed in a broad range and their distribution patterns were quite different between the strains isolated during 1980 to 1984, and 1994 to 1995, as shown in Table 1. For example, most of the strains isolated during 1980 to 1984 were susceptible to OTC at 0.78 µg/ml or less (MIC₉₀; 0.78 µg/ml), while strains isolated during 1994 to 1995 were susceptible at more than 0.78 µg/ml (MIC₉₀; 3.13 µg/ml). Similar differences in the distribution patterns of MICs of CTC were also observed between strains isolated in the different periods. There were no differences in MICs between strains isolated from the lung and those of synovia.

Strains, IS-1, which was isolated from the lung in 1995, and IS-2, isolated from the synovia in 1994, showed resistance to all macrolides tested and LCM.

***M. hyorhinis*:** The distribution patterns of MICs obtained

from 108 strains of *M. hyorhinis* were similar to those obtained with *M. hyosynoviae* (Table 2). For example, among the 16-membered macrolides, JM, TS, and KT had higher activities than SPM, and for the 14-membered macrolides, EM had no effect on strains of *M. hyorhinis*.

Eleven out of the 108 strains tested showed resistance to all macrolides tested and LCM. These macrolides-resistant strains were isolated from the lung, thoracic fluid, pericardial fluid, abdominal fluid, or kidney of 4 piglets with polyserositis derived from 2 areas.

Susceptibility of *M. hyosynoviae* and *M. hyorhinis* to KM, CP or TP was of the secondary grade. CP and TP showed the same activity to both mycoplasmas (MIC₉₀; 3.13 µg/ml, respectively). However, *M. hyorhinis* susceptibility to KM was higher (MIC₉₀; 3.13 µg/ml) than that of *M. hyosynoviae* (MIC₉₀; 25 µg/ml).

DISCUSSION

The results of the present study demonstrated impressive *in vitro* effectiveness of TML, LCM, 16-membered macrolides (JM, TS, and KT), tetracyclines and ERFX tested against 54 Japanese strains of *M. hyosynoviae* and 107 Japanese strains of *M. hyorhinis* collected in the last 15 years.

The macrolides are commonly classified into 2 groups, by their chemical structure [2], either as the 14-membered or the 16-membered group. It has been suggested that the 14-membered macrolides, such as EM, are very effective against human mycoplasmal pneumonia caused by *M. pneumoniae* [15]. However, EM susceptibility varies widely among the mycoplasma species [15]. Additionally, the 16-membered macrolides seem more effective against *Mollicutes* than the 14-membered macrolides [5, 8, 14, 18]. According to the results of *M. hyosynoviae* and *M. hyorhinis* susceptibility to EM, a 14-membered macrolide, EM was not active. Oleandomycin, also a 14-membered macrolide, showed similar results to EM when tested against both mycoplasmas (data not shown). We also found that 2 field-isolates of *M. hyosynoviae* and 11 field-isolates of *M. hyorhinis* were resistant to all the macrolides tested. The presence of plasmid encoding for macrolide-resistance is unlikely since we have not been able to detect any plasmids in the macrolide-resistant strains (data not shown). Ribosomal protein alteration [12, 19] and alternations in ribosomal RNA [16] are the resistant mechanisms against macrolides in *Escherichia coli*. In addition, structural modification of EM by phosphorylation [10], glycosylation [4], and lactone ring cleavage by EM esterase [11] have recently been reported as the resistant mechanisms of many bacterial species against EM. However, the mechanisms of resistance to the macrolides in *Mollicutes* are not clear. Only one report describes the resistant mechanisms of *M. pneumoniae* against EM [9]; that is transition mutations in the 23S rRNA [9]. In a previous study, we confirmed inducible resistance to 16-membered macrolides in 2 field isolates of *M. hyorhinis* [6]. We consider that the 16-

Table 1. Antimicrobial agent susceptibility of 55 strains of *M. hyosynoviae*

Drug	Year of isolation	Minimum inhibitory concentration ($\mu\text{g/ml}$)															MIC ₉₀ $\mu\text{g/ml}$
		>100	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.2	0.1	0.05	0.025	0.013	
JM	1980–1984										13 ^{a)}	15 ^{*b)}					0.39
	1994–1995				1	1					9	16					0.39
TS	1980–1984									3	3	6	14*	2			0.78
	1994–1995				2					4	5	7	7	2			0.78
SPM	1980–1984						2	26*									6.25
	1994–1995		1	1			2	23									6.25
KT	1980–1984										9*	19					0.39
	1994–1995				2						10	15					0.39
EM	1980–1984	22*	5	1													>100
	1994–1995	15	12														>100
LCM	1980–1984										12	16*					0.39
	1994–1995				2						6	18	1				0.39
KM	1980–1984				6	11	6*	4	1								25
	1994–1995				5	12	7	3									25
CP	1980–1984							14	11*	3							3.13
	1994–1995							6	9	2							3.13
TP	1980–1984							20	8*								3.13
	1994–1995					1	19	7									3.13
TML	1980–1984												1	18*	8	1	0.05
	1994–1995													14	12	1	0.05
OTC	1980–1984								6	19*	2	1					0.78
	1994–1995					1	2	19	5								3.13
CTC	1980–1984								11	3	13*	1		2			1.56
	1994–1995					9	12	6						2			6.25
ERFX	1980–1984										14*	14					0.39
	1994–1995										18	9					0.39

a) Number of strains.

b) The asterisk mark indicates that the group includes type strain S16.

membered macrolide resistant mycoplasmal strains obtained in the present study will be useful for clarifying the macrolide resistant mechanisms.

A few reports describe macrolide resistant mycoplasmas: TS and SPM resistant *M. gallisepticum* (14 of 27 strains tested; 52%) isolated from Japanese chickens and unhatched eggs [7]; and EM resistant *M. gallisepticum* (46 of 112 strains tested; 41%) isolated from Japanese chickens [14]. This high ratio of appearance of resistance to macrolides could be due to antimicrobial agents having been overused to control bacterial diseases in Japanese chickens. Recent changes in pig farm management are making them more like the intensive poultry industry. Not only macrolides, but also other kinds of antimicrobial agents might be ineffective against porcine mycoplasmas. Yamamoto *et al.* [20] have reported that the MICs of tetracyclines (TC, OTC,

and doxycycline) to *M. hyopneumoniae* strains isolated in 1970 were 0.04 to 0.63 $\mu\text{g/ml}$, but were 0.08 to 2.5 $\mu\text{g/ml}$ for these isolated in 1980, and similar results were also observed by Etheridge *et al.* [1] and Inamoto *et al.* [3]. In the present study, we observed that *M. hyosynoviae* strains isolated during 1994 to 1995, from the lungs or synovia, were becoming resistant to tetracyclines (OTC and CTC), the MICs obtained from these strains being higher than those of the strains which had been isolated in 1980 to 1984. We could not observe previously isolated strains of *M. hyorhinis*, however, recently isolated *M. hyorhinis* strains from piglets also seemed to be becoming resistant to tetracyclines, since the MICs of more than 70% of *M. hyorhinis* tested were higher than those of type strain BTS7 which was isolated about 40 years ago. Furthermore, two peaks (3.13 and 0.2 to 0.8 $\mu\text{g/ml}$) were observed in MICs distribution in each of

Table 2. Antimicrobial agent susceptibility of 108 strains of *M. hyosynoviae*

Drug	Minimum inhibitory concentration ($\mu\text{g/ml}$)												MIC ₉₀ $\mu\text{g/ml}$
	>100	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.2	0.1	
JM		1 ^{a)}	10					25	38* ^{b)}	26	8		50
TS			1	2	8			6	81*	10			12.5
SPM	11				3	9	62*	21	2				>100
KT		2	9					8	60	29*			50
EM	108*												>100
LCM			11					32*	65				100
KM						3	11	53	40*	1			3.13
CP							14	65*	24	5			3.13
TP						9	76*	18	5				3.13
TM									7	44*	57		0.39
OTC						14	25	25	15	10*	19		6.25
CTC						15	38	9	15*	14	17		6.25
ERFX									64*	44			0.78

a) Number of strains.

b) The asterisk mark indicates that the group includes type strain BTS7.

the tetracyclines. Therefore, MICs of tetracyclines against isolates of *M. hyorhinis* should show a broad distribution (0.2 to 6.25 $\mu\text{g/ml}$).

The increasing resistance of Japanese strains of porcine pathogenic mycoplasmas (*M. hyosynoviae*, *M. hyorhinis*, and *M. hyopneumoniae*) to tetracyclines may reflect that tetracyclines, especially CTC, have been much more widely used than other antimicrobial agents for veterinary use in Japan.

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