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Macrolides and Lincomycin Susceptibility of *Mycoplasma hyorhinis* and Variable Mutation of Domain II and V in 23S Ribosomal RNA

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ABSTRACT. A total of 151 strains of *Mycoplasma hyorhinis* isolated from porcine lung lesions (weaned pigs, n = 71, and finishers, n = 80) were investigated for their *in vitro* susceptibility to 10 antimicrobial agents. Thirty-one strains (28 from weaned pigs and 3 from finishers) showed resistance to 16-membered macrolide antibiotics and lincomycin. The prevalence of the 16-membered macrolide-resistant *M. hyorhinis* strain in weaned pigs from Japanese herds has approximately quadrupled in the past 10 years. Several of the 31 strains were examined for mutations in the 23S ribosomal RNA (rRNA). All field strains tested showed a transition of A to G at position 2059 of 23S rRNA-rendered *Escherichia coli*. On the other hand, individual tylosin- and lincomycin-resistant mutants of *M. hyorhinis* were selected *in vitro* from the susceptible type strain BTS7 by 3 to 9 serial passages in subinhibitory concentrations of each antibiotic. The 23S rRNA sequences of both tylosin and lincomycin-resistant mutants were compared with that of the radical BTS7 strain. The BTS7 mutant strain selected by tylosin showed the same transition as the field-isolated strains of A2059G. However, the transition selected in lincomycin showed mutations in domains II and V of 23S rRNA, G2597U, C2611U in domain V, and the addition of an adenine at the pentameric adenine loop in domain II. The strain selected by lincomycin showed an additional point mutation of A2062G selected by tylosin.

KEY WORDS: antimicrobial susceptibility, macrolide resistance, *Mycoplasma hyorhinis*, 23S ribosomal RNA, tylosin.

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Mycoplasma hyorhinis, which causes arthritis, pleuritis, peritonitis, pericarditis, and polyserositis, is frequently isolated from the upper respiratory tract and tonsils of young pigs [3, 4]. It is also reported as a causal agent of otitis media [13]. It has often been isolated from pigs with porcine reproductive and respiratory syndrome (PRRS) [8, 19]. No vaccines have been available to control the spread of *M. hyorhinis* within herds. Thus, at present, chemotherapeutic control is the most practical way to minimize the economic losses caused by *M. hyorhinis* infection. There have been several reports examining the susceptibility of *M. hyorhinis* to antimicrobial agents [1, 2, 6, 9, 10, 20, 24]. Over the course of a decade in Japan, we detected isolates of *M. hyorhinis* and *M. hyosynoviae* that were resistant to 16-membered macrolides, such as tylosin and josamycin [9, 10]. A few reports describe macrolide resistance in swine mycoplasmas in European countries [1, 6]; however, no macrolide-resistant *M. hyorhinis* has been detected in the US. [24]. Alterations in the ribosomal proteins of L4 and L22 [16, 23] and rRNA [22] have been reported as the mechanism of resistance to macrolides in *Escherichia coli*. In addition, structural modifications of erythromycin by phosphorylation [14], glycosylation [7], and lactone ring cleavage by erythromycin esterase [15] have been reported as the mechanisms of resistance of many bacterial species to erythromycin. The macrolide-binding site is composed primarily of RNA. Segments of 23S rRNA, the central loop of domain V, and the loop of hairpin 35 from domain II are believed to be the major components of the drug binding site on the ribosome [5]. Lucier *et al.* [12] were the first to report the mechanism of erythromycin-resistant *M. pneumoniae*, the causal agent of human mycoplasmal pneumonia, in which 23S rRNA gene encoding domain V revealed an A-to-G transition in the central loop at position 2063 or an A-to-G transition at position 2064. Recently, Pereyre *et al.* [17, 18] have further analyzed the mechanism of resistance in the human mycoplasmas *M. hominis, M. fermentans* and *M. pneumoniae*. They found other transitions in the domain V sequences in 16-membered macrolide-resistant mycoplasmas that confer intrinsic resistance to 14- and 15-membered macrolides. Furthermore, no interfering target sites of macrolides by methylation or mutation of ribosomal protein (L4 or L22) were found in these human mycoplasmas [17]. Thus, mycoplasmal resistance to macrolides appears to depend on the mycoplasma species and mutation levels in 23S rRNA.

The resistance mechanism to macrolides of human-originating mycoplasmas has been analyzed; however, little is known about animal versions. Since huge quantities of antimicrobial agents, including macrolides and lincomycin, are used in the Japanese swine industry as growth promoters and for disease control, macrolide-resistant *M. hyorhinis* and *M. hyosynoviae* strains are often isolated from weaned pigs [10]. In the present study, we investigated the *in vitro* activities of 10 antimicrobial agents that are commonly available for veterinary use in Japan against *M. hyorhinis* field isolates from both weaned pigs and finishers in slaughterhouses. In addition, to analyze the mechanism of mac-

rolide resistance in *M. hyorhinis*, macrolide-resistant mutant BTS7^T (T = type strain) and field strains showing resistance to these macrolide agents were examined for 23S rRNA transitions.

MATERIALS AND METHODS

Mycoplasma strains and growth conditions: The type strain of *M. hyorhinis* BTS7 was derived from the stock culture collection of the National Institute of Animal Health. The IR-2 and IR-6 reference strains, which are resistant to 16-membered macrolides, have been reported previously [9]. A total of 151 *M. hyorhinis* strains were collected from lung lesions (71 strains from 71 piglets around 2–3 months old from 25 farms and 80 strains from 80 slaughtered pigs from 51 farms in various parts of Japan) between May 2002 and April 2004. All the strains were incubated in PPLO broth (Difco) with 0.5% mucin bacteriological (Difco) extract (M-base) supplemented with 15% (v/v) heat-inactivated horse serum, 3% (v/v) of 25% (w/w) fresh yeast extract (M-broth), and its solid medium (1.2% (w/v) noble agar (Difco), M-agar) [9]. Briefly, M-base was prepared by placing 5 g mucin and 21 g PPLO broth in 840 m*l* distilled water and heating it in a boiling water bath for 20 min. The solution was filtered using a NA900 filter system (Advantic, Japan) after centrifugation at 10,000 × g for 15 min. The pH of M-broth is usually around 7.6, so no pH adjustment is necessary.

Drugs and the antimicrobial susceptibility test: The following 10 antimicrobial agents, which are approved for therapeutic use in the porcine industry in Japan, were used: tylosin, josamycin, lincomycin, tiamulin, kanamycin, thiamphenicol, oxytetracycline, enrofloxacin, kitasamycin and spiramycin. Kitasamaycin was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan, and the other drugs were supplied by the National Veterinary Assay Laboratory, Tokyo, Japan. Serial two-fold dilution of the drugs was carried out in test tubes with M-broth. One milliliter of each dilution was mixed with 9 m*l* of M-agar. M-agar plates without antimicrobial agents served as the controls. Aliquots of the organisms (5 μl, containing approximately 10^6 CFU/m*l*) were taken from the logarithmic phase and inoculated onto plates with a microplanter (Sakuma Co., Ltd., Tokyo, Japan). The plates were incubated at 37°C with 5% CO_2 gas in air. MICs were read 4 days after inoculation. The lowest concentration of a drug to inhibit 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. Since no break point of MICs have been established for mycoplasma species, the bullet value of bimodal distribution of each drug was defined as the break point.

Selection of macrolide-resistant mutants of M. hyorhinis BTS7: Selection of macrolide-resistant mutants of *M. hyorhinis* BTS7 was carried out in the same way as previous study [17]. The BTS7 strain was developed by serial transfer in M-broth containing subinhibitory concentrations of tylosin or lincomycin. In the first passage, the BTS7 strain was incubated in M-broth with successive two-fold dilution of each drug. The MIC of each passage was determined as the lowest concentration of the drug that prevented growth in the broth at the same time as the drug-free growth control first showed growth (approximately 3 days after incubation at 37°C). When the BTS7 strain selected by lincomycin or tylosin showed a steady MIC value or MIC over 100 μg/m*l*, respectively, 3 colonies from each drug-containing medium were selected and their MIC confirmed against macrolides and lincomycin. Domains II and V of their 23S rRNA genes were then examined.

DNA sequence of 23S rRNA in M. hyorhinis BTS7 and PCR amplification of domain II and V: Since the 23S rRNA sequence of *M. hyorhinis* is considered to have high homology with that of *M. hyopneumoniae* (accession no. GI 288499), several putatively conservative sequences in 23S rRNA of *M. hyopneumoniae* were selected and oligonucleotide primers were prepared after alignment with the 23S rRNA sequence of *M. hominis* that was previously reported in GeneBank (accession no. GI 23307625). Most of the 23S rRNA sequences (approx. 2500 bp) of the BTS7 strain, including the complete domain II and V sequences, were decided in this study (accession no. AB182581). Specific primer pairs for amplification of domains II and V of 23S rRNA in *M. hyorhinis* were selected from the sequences obtained from the present study. The primer sequences designated for each domain were as follows: domain II, Mhr-D2F 5'-ATCCATGAGCAGGTTGAAGC-3' (at 23S rRNA position 722 in *Escherichia coli*) and Mhr-D2R 5'-CCATTCCACATTCAGTGCTC-3' (at position 886 in *E. coli*), amplicon size of 193 bp; domain V, Mhr-D5-1F 5'-CACGAAAGGCGCAATGATCTC-3' (at position 1999 in *E. coli*) and Mhr-D5-1R 5'-CACTAGAACTAGCGTCCCAGC-3' (at position 2189 in *E. coli*), amplicon size of 192 bp; Mhr-D5-2F 5'-CTCATCGCATCCTGGAGCTG-3' (at position 2542 in *E. coli*) and Mhr-D5-2R 5'-CCGCTTAGATGCTTTCAGCG-3' (position at 2795 in *E. coli*), amplicon size of 254 bp. The amplicons, purified by 2% agarose gel-electrophoresis, were sequenced with an ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit and an ABI Prism 377 Sequencer (Applied Biosystems), following the manufacturer's instructions.

DNA sample preparation and PCR: One m*l* of well-grown culture including the selector drug (20 μg/m*l* of tylosin or lincomycin) was centrifuged at 10,000 × g for 10 min. After washing the pellet with 1 m*l* PBS, the pellet was resuspended in a 100 μl aliquot of lysis buffer containing 150 μg/m*l* proteinase K (Sigma) in PBS, incubated at 57°C for 1 hr, heated in a boiling water bath for 5 min to inactivate the proteinase K, and then centrifuged at 10,000 × g for 10 min. The supernatant was used as the PCR template. Amplification was performed in a total volume of 100 μl containing 10 mM Tris-HCl (pH 8.3 at 25°C), 1 mM $MgCl_2$, 50 mM KCl, 100 μM dNTP, 2.5 units of rTaq DNA polymerase (Toyobo, Japan), 200 nM of each primer, and 2 μl of template DNA. The PCR assay was carried out in a Perkin

Table 1. MIC for *Mycoplasma hyorhinis* stranis isolated from both weaners and finishers by the agar dilution

Antimicrobials	MIC (μg/ml)[a] for field strains isolated						
	Weaned pigs (n=71)			Finishers (n=80)			Type strain BTS7
	50%	90%	Range	50%	90%	Range	
Tylosin	0.8	100	0.8–100	0.8	1.56	0.4–25	0.8
Josamycin	0.8	50	0.2–50	0.4	1.56	0.2–25	0.8
Kitasamycin	0.8	50	0.2–50	0.4	0.8	0.4–25	0.8
Lincomycin	0.8	>100	0.8–>100	0.8	1.56	0.4–50	1.56
Tiamulin	0.8	1.56	0.2–1.56	0.4	0.8	0.2–1.56	0.4
Kanamycin	6.25	12.5	0.8–25	3.13	6.25	0.8–25	0.8
Thiamphenicol	3.13	6.25	1.56–12.5	3.13	6.25	1.56–12.5	3.13
Oxytetracycline	0.2	0.4	0.1–0.4	0.2	0.4	0.2–0.8	0.4
Enrofloxacin	0.4	1.56	0.2–1.56	0.4	1.56	0.2–1.56	0.8

a) 50% and 90%, MIC at which 50 and 90% of the strains tested, respectively, are inhibited.

Elmer 2400 Thermocycler, comprising 2 min of pre-incubation at 94°C, followed by 30 cycles of 30 sec at 94°C, 45 sec at 60°C, and 1 min at 72°C. Final extension was performed for 7 min at 72°C. DNA from the BTS7 strain was used as the positive control template. The strains originating from BTS7 subcultured from passages with the selector drugs (tylosin or lincomycin), IS-2 and IS-6 strains, and 4 isolates from weaned pigs and 2 isolates from finishers showing resistance to the 16-membered macrolide agents in this study were characterized by amplification and sequencing.

RESULTS

Antimicrobial susceptibility of field-isolated strains: The distribution of the MICs obtained from the 151 strains tested is given in Table 1. The MIC_{50} results obtained from the weaned pigs and finishers were almost the same, but the MIC_{90} results were different for 16-membered macrolides, such as tylosin and josamycin. Oxytetracycline showed the lowest MIC (high activity), 0.1–0.4 and 0.2–0.8 μg/m*l* for weaned pigs and finishers, respectively. Tiamulin and enrofloxacin showed higher activity than 16-membered macrolides. The BTS7 strain showed sensitivity to 0.8 μg/m*l* kanamycin; however, all field strains showed lower susceptibility to kanamycin than the BTS7 strain. Of the 71 strains from weaned pigs and the 80 strains from finishers, 28 strains and 3 strains, respectively showed resistance to macrolides and lincomycin. The details of MICs for these 31 strains, which showed macrolide or lincomycin resistance, are listed in Table 2. All 31 strains that showed resistance to 16-membered macrolides were also resistant to lincomycin, and vice versa.

Macrolides and lincomycin susceptibility of resistant mutants of M. hyorhinis BTS7: MICs of the BTS7 strain were shown to have changed during serial subculture in M-broth with tylosin or lincomycin (Fig. 1). Resistance to tylosin in BTS7 could be selected after 3 passages and resistance to lincomycin after 6 passages. Both resistant BTS7 strains showed steady MIC values of 100 μg/m*l* after 5 tylosin passages and of 50 μg/m*l* after 9 lincomycin passages. The selected MIC for tylosin of resistant BTS7 strains after 5 tylosin passages was more than 100 μg/m*l*; however, the selected MIC for tylosin after 9 lincomycin

Table 2. Macrolide susceptibility of 31 *Mycoplasma hyorhinis* strains showing resistance to macrolides and lincomycin

Macrolide	Minimum inhibitory conc. (μg/m*l*)				
	12.5	25	50	100	>100
Lincomycin		1[a](1)[b]	2 (2)		28
Tylosin		10 (3)	18	3	
Josamycin	2	13 (3)	16		
Kitasamycin	4	10 (3)	17		
Spiramycin			3 (3)	28	

a) Number of strains.
b) Number of strains isolated from finishers.

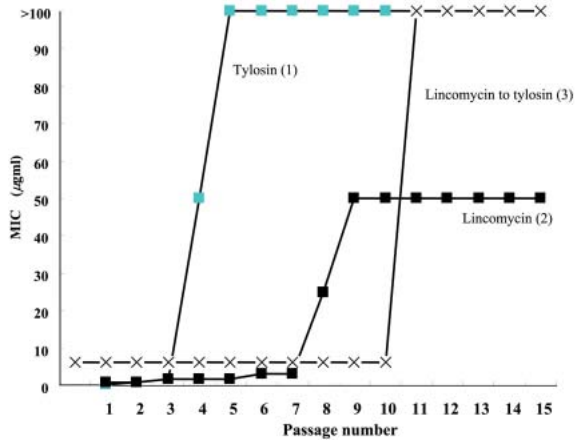

Fig. 1. Change in the MICs of *M. hyorhinis* BTS7 during subculture and serial transfer in both tylosin and lincomycin. *M. hyorhinis* BTS7 to tylosin subcultured in subinhibitory concentrations of tylosin (1), MICs of BTS7 to lincomycin with subinhibitory concentrations of lincomycin (2), and MICs of lincomycin-resistant BTS7 to tylosin with subinhibitory concentrations of tylosin (3).

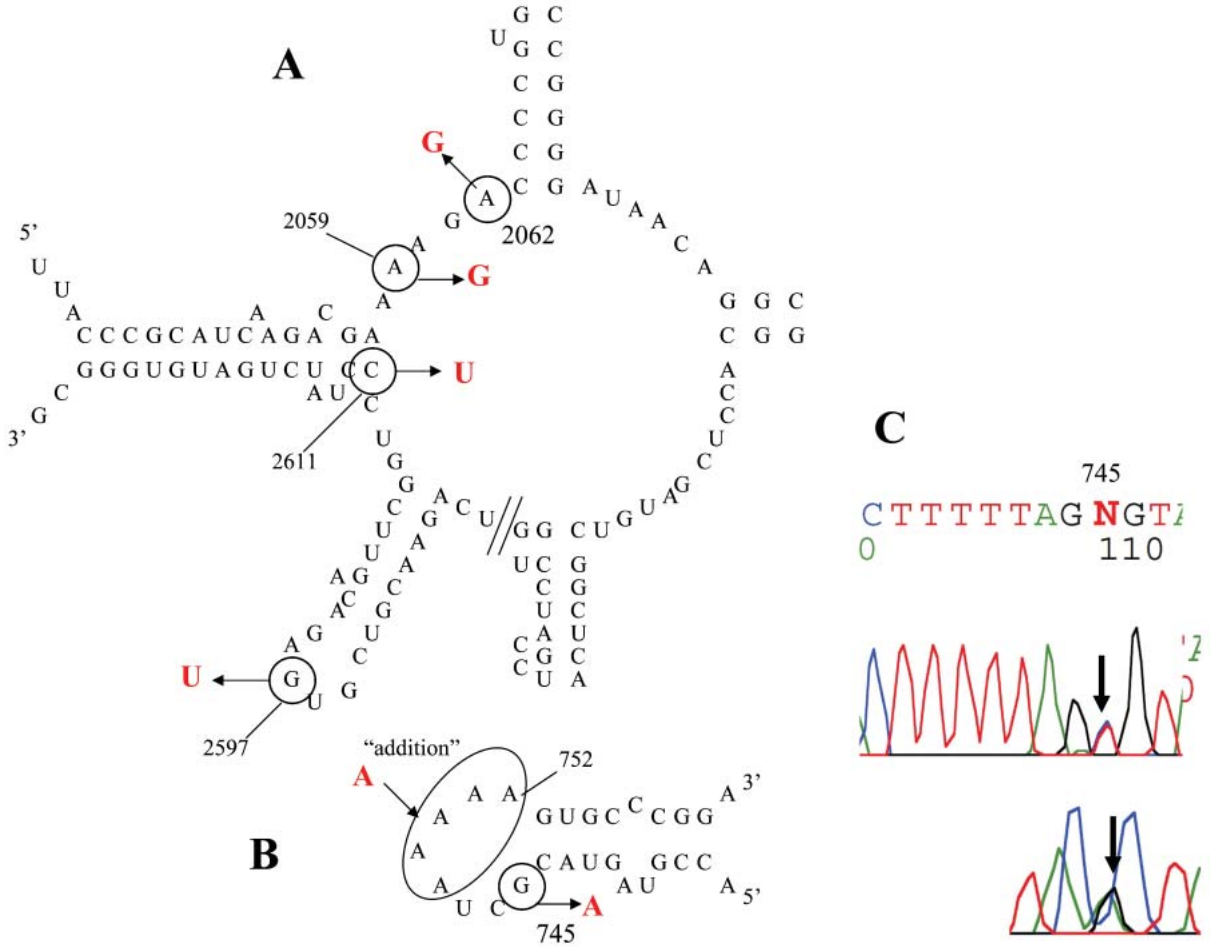

Fig. 2. Secondary structure of the center of domain V (A) of 23S rRNA and hairpin 35 of domain II (B) from *M. hyorhinis* BTS7. The beginning of tylosin-resistant BTS7 (at 3 passages) showed G745A in domain II (B, C). The nucleotides are numbered on the basis of *E. coli* sequences.

passages was 6.25–12.5 μg/m*l*. These resistant mutant strains showed approximately the same MIC values for the other 16-membered macrolides (josamycin, spiramycin, and kitasamycin) tested.

Mutation of domains II and V in 23S rRNA in macrolide and lincomycin-resistant strains: A total of 11 strains, comprising 3 strains of mutant BTS7 selected by tylosin or lincomycin, IS-2 and IS-6, and six 16-membered macrolide-resistant strains, 4 from weaned pigs and 2 from finishers, were analyzed by amplification and sequencing of domain II and V. Domain sequences and mutations obtained from resistant strains are illustrated in Fig. 2. The mutant BTS7 strain that developed lincomycin resistance on exposure to lincomycin (Fig. 1, 15 passages) showed two point mutations, at positions G2597U and C2611U in domain V, and the addition of an adenine at pentameric adenine sequences in domain II. At the third passage, the BTS7 strain (Fig. 1, MIC 1.6 μg/m*l*) selected by tylosin showed a one-point mutation at G745A in domain II. However, it contained both G745 and A745 before tylosin resistance developed (Fig. 2C, arrows indicated). On the other hand, a completely tylosin-resistant mutant BTS7 strain (Fig. 1, 15 passages) showed only a one point mutation at position A2059G, with no G745A mutation confirmed. All field-isolated strains, including IS-2 and IS-6, showed the same point mutation of A2059G as the completely tylosin-resistant BTS7 strain.

Furthermore, the mutant BTS7 strain developed lincomycin resistance accompanied by moderate tylosin resistance (MIC of 6.25 μg/m*l*, at 15 passages), when selected by tylosin, as described previously. After 11 tylosin passages by serial transfer, the lincomycin-resistant BTS7 strain showed higher resistance (>100 μg/m*l*) to tylosin (Fig. 2). The mutation of the mutant BTS7 strain in 23S rRNA was A2062G, in addition to G2597U and C2611U, which had been selected by lincomycin. The characteristic mutation for tylosin resistance, A2059G, was not observed.

DISCUSSION

When *M. hyorhinis* strains were isolated from weaned pigs in Japan a decade ago approximately 10% of them showed resistance to 16-membered macrolides and lincomycin [9]. However, in this study, nearly 40% of strains from weaned pigs were resistant to these drugs. The susceptibility of recent isolates to oxytetracycline was higher than those of former isolates, which had an MIC range of 0.2 to 6.3 μg/m*l* [9, 10]. We believe that these contrasts might be a result of the extensive use of antimicrobial agents for disease control. Because many weaned pigs in large herds in Japan often suffer respiratory syndrome, antimicrobial agents, especially macrolides, are the first choice drug for disease control. However, finishers are usually not exposed to macrolide drugs, since the main bacterial causal agent of respiratory disease for them is increasingly *Actinobacillus pleuropneumoniae* [21], against which macrolide drugs are not effective. On the other hand, 17 antimicrobial drugs, including tylosin, kitasamaycin, and chlortetracycline have long been licensed for use in the Japanese pig industry as antimicrobial growth promoters (AGPs), but no strains of antimicrobial-resistant *M. hyorhinis*, except for 16-membered macrolide drugs, for AGP drugs were reported. Isolates resistant to 16-membered macrolides were approximately 4 times more common than observed in our previous study [9, 10]. This fact suggests that the use of AGPs is not responsible for making *M. hyorhinis* resistant to AGP drugs. We also concluded that due to the appearance of many tetracycline-resistant pathogens [11], use of tetracyclines might be decreasing in the pig industry with the result that, in Japan, there might be an increased use of enrofloxacin as an alternative. Co-selection of 16-membered macrolide drugs for therapeutic use in weaned pigs with respiratory symptoms was probably an important factor in maintaining the level of macrolide-resistant *M. hyorhinis* is approximately 40%. On the other hand, macrolide-resistant *M. hyorhinis* accounted for only 3 out of 80 (3.8%) strains from finishers in slaughterhouses. We believe that some strains with macrolide resistance may have reverted to being susceptible to macrolides *in vivo* because most finishers after 3 to 4 months old are not treated with drugs for disease control. Our previous study [9] confirmed that macrolide-resistant *M. hyorhinis* strains can revert to susceptible types by *in vitro* passage in antimicrobial-free broth medium. If this were not the case, macrolide-sensitive strains would have been present in large numbers in the *M. hyorhinis* populations of finisher pigs. Furthermore, the loop-structure of ribosomal RNA easily mutates regardless of the presence of antimicrobial agents. If a point mutation accidentally occurred at an important region for the working point of macrolide drugs, the mutant strain should be selected under the macrolide existent condition.

The resistance of *M. hyorhinis* could be more rapidly selected *in vitro* by tylosin (several serial passages) and lincomycin (in under 10 passages), than could be seen with *M. pneumoniae* [18]. Since *M. hyorhinis* has intrinsic resistance to 14-membered macrolides (erythromycin and oleandomycin) [9, 10], it might be easier for *M. hyorhinis* to acquire resistance to macrolides other than 14-membered macrolides. If *M. hyorhinis* acquires macrolide resistance in the wild as well as *in vitro*, macrolide treatment of pigs needs to be more rigorous in farm environments for the reasons described above: the 16-membered macrolides of tylosin and josamycin, as well as lincomycin, are the most commonly used as therapeutic treatment for porcine mycoplasmosis in Japanese herds. In addition, the dosing period of drugs has needed an extension due to PRRS virus infection.

Although various mutations occurred in domains II and V of 23S rRNA in the lincomycin-resistant BTS7 strain (Fig. 1 (2)), the MIC value of this strain to tylosin was not very high (6.25 μg/m*l*). On the other hand, the BTS7 strain selected by tylosin (Fig. 1 (1)) showed resistance to lincomycin at the same MIC level as the lincomycin-selected resistant BTS7 strain (Fig. 1 (2)). However, when the lincomycin-selected resistant BTS7 strain (Fig. 1 (2)) was selected by tylosin, a point mutation (A2062G) was caused, resulting in showing a MIC value of greater than 100 μg/m*l* to tylosin. In addition to these results, it was revealed that a single A2059G mutation, observed in all tylosin-resistant strains selected by some of the wild or *in vitro* strains, could lead to a high level of resistance to both tylosin and lincomycin. Therefore, point mutations at positions A2059G or A2062G in 23S rRNA appear to play a very important role in conferring resistance to 16-membered macrolides and lincomycin. Previous reports [5, 12, 17, 18] have focused on the relationship between macrolide resistance and an alteration in 23S rRNA, and thus point mutations, in particular, at a position between 2058 and 2062 (the positions in *E. coli*) in domain V of 23S rRNA appear to be important. In our continuous study of this, an *in vitro* selected-tylosin resistant strain of *M. hyopneumoniae* (J^T strain; MIC of > 50 μg/m*l* to tylosin) also shows a few point mutations in the same region (detailed data are being compiled). The reason that the lincomycin-resistant BTS7 strain (Fig. 1 (2)) did not show a high level of resistance to tylosin may be that no alteration occurred between 2058 and 2062 in domain V. Interestingly, although two point mutations, G2597U and C2611U, developed in domain V in cases where resistance was primarily selected by lincomycin, a single point mutation of A2058G did not occur, even though the strain was successively selected by tylosin (tylosin resistance was acquired by a point mutation of A2062G, as previously described). Although the reason for this phenomenon is unknown, we believe that various mutations occurring in domain V in the lincomycin-selected resistant strain have an influence on the formation of the tertiary structure of 23S rRNA and the binding of 23S rRNA to the 50S subunit in ribosome [5]. Mutations caused by exposure to an antimicrobial agent (lincomycin) that prevent the agent from binding to 23S rRNA may lead to a disturbance of the physical structure of 23S rRNA, originally possessed by the susceptible strain, resulting in a failure to cause the most effective point mutation, A2058G. Although the lincomycin-selected resistant

strain can eventually survive exposure to tylosin by acquiring the A2062G mutation, the number of passages needed to cause this mutation may also be one of the reasons for the failure to obtain the A2058G mutation.

In conclusion, the prevalence of 16-membered macrolide-resistant *M. hyorhinis* in weaned pigs has quadrupled in Japanese herds over the last 10 years. The increased prevalence of this resistant bacterium is not attributable to the use of AGP: we believe that it has been caused by multiple use of antimicrobial agents as chemotherapy. The difference between tylosin-resistant and -susceptible strains of *M. hyorhinis* BTS7 was only A2059G in domain V of 23S rRNA. However, lincomycin showed a variable modification in both domain II and V, and lincomycin-resistant *M. hyorhinis* strains showed slow development of resistance to tylosin. Therefore, lincomycin should be the first choice for treatment of diseases caused by organisms that are susceptible to both macrolides and to lincomycin.

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