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 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.
rolide resistance in *M. hyorhinis*, macrolide-resistant mutant BTS7 (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 ml 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 x 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. Kitasamycin 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 aliquot of the organisms (5 µl, containing approximately 10^6 CFU/ml) 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% CO₂ 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/ml, 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. hypopneumoniae* (accession no. GI 288499), several putatively conservative sequences in 23S rRNA of *M. hypopneumoniae* 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’-CCAT-TCCACATTCTCAGTC-3’ (at position 886 in *E. coli*), amplicon size of 192 bp; domain V, Mhr-D5-1F 5’-CAC-GAAAGGGCGCAATGATCTC-3’ (at position 1999 in *E. coli*) and Mhr-D5-1R 5’-CAGCATTGATCTC-3’ (at position 2189 in *E. coli*), amplicon size of 192 bp; domain V, Mhr-D5-2F 5’-CTCATCGCATCCTGGAGCTG-3’ (at position 2542 in *E. coli*) and Mhr-D5-2R 5’-CCGCTTATGGTTTGCTG-3’ (at position 2795 in *E. coli*), amplicon size of 254 bp. The amplimers, 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 ml of well-grown culture including the selector drug (20 µg/ml of tylosin or lincomycin) was centrifuged at 10,000 x g for 10 min. After washing the pellet with 1 ml PBS, the pellet was resuspended in a 100 µl aliquot of lysis buffer containing 150 µg/ml 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 x 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 MgCl₂, 50 mM KCl, 100 µM dNTP, 2.5 units of Taq DNA polymerase (Toyobo, Japan), 200 nM of each primer, and 2 µl of template DNA. The PCR assay was carried out in a Perkin
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 MIC50 results obtained from the weaned pigs and finishers were almost the same, but the MIC90 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/ml 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/ml 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/ml after 5 tylosin passages and of 50 µg/ml after 9 lincomycin passages. The selected MIC for tylosin of resistant BTS7 strains after 5 tylosin passages was more than 100 µg/ml; however, the selected MIC for tylosin after 9 lincomycin

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

<table>
<thead>
<tr>
<th>Antimicrobials</th>
<th>MIC (µg/ml)a) for field strains isolated</th>
<th>Type strain</th>
<th>BTS7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weaned pigs (n=71)</td>
<td>Finishers (n=80)</td>
<td></td>
</tr>
<tr>
<td>Tylosin</td>
<td>0.8 100 0.8–100</td>
<td>0.8 1.56 0.4–25</td>
<td>0.8</td>
</tr>
<tr>
<td>Josamycin</td>
<td>0.8 50 0.2–50</td>
<td>0.4 1.56 0.2–25</td>
<td>0.8</td>
</tr>
<tr>
<td>Kitasamycin</td>
<td>0.8 50 0.2–50</td>
<td>0.4 0.8 0.4–25</td>
<td>0.8</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>0.8 &gt;100 0.8–100</td>
<td>0.8 1.56 0.4–50</td>
<td>1.56</td>
</tr>
<tr>
<td>Tiamulin</td>
<td>0.8 1.56 0.2–1.56</td>
<td>0.4 0.8 0.2–1.56</td>
<td>0.8</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>6.25 12.5 0.8–25</td>
<td>3.13 6.25 0.8–25</td>
<td>0.8</td>
</tr>
<tr>
<td>Thiapenicol</td>
<td>3.13 6.25 1.56–12.5</td>
<td>3.13 6.25 1.56–12.5</td>
<td>3.13</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>0.2 0.4 0.1–0.4</td>
<td>0.2 0.4 0.2–0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>0.4 1.56 0.2–1.56</td>
<td>0.4 1.56 0.2–1.56</td>
<td>0.8</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Macrolide</th>
<th>Minimum inhibitory conc. (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12.5</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>1</td>
</tr>
<tr>
<td>Tylosin</td>
<td>10</td>
</tr>
<tr>
<td>Josamycin</td>
<td>2</td>
</tr>
<tr>
<td>Kitasamycin</td>
<td>4</td>
</tr>
<tr>
<td>Spiramycin</td>
<td>3</td>
</tr>
</tbody>
</table>

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).
passages was 6.25–12.5 µg/ml. 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/ml) 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/ml, 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/ml) 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.

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.
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/ml [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, kitasamycin, and chlorotetracycline 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 loss of many tetracycline-resistant pathogens [11].

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/ml). 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/ml 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* strain; MIC of > 50 µg/ml 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 \textit{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 \textit{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 \textit{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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