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Characterization of *In Vivo* Acquired Resistance of *Mycoplasma hyopneumoniae* to Macrolides and Lincosamides

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ABSTRACT

Macrolides and related antibiotics are used to control mycoplasma infections in the pig industry worldwide. Some porcine mycoplasmas, however, survive these treatments by acquiring resistance. The mechanism of acquired resistance to macrolides and lincosamides was studied in more detail for *Mycoplasma hyopneumoniae* by comparing both the phenotype and genotype of a resistant field isolate to five susceptible isolates. The MICs were significantly higher for the resistant strain for all antibiotics tested. The MICs for the 16-membered macrolide tylosin ranged from 8 to 16 μg for the resistant strain and from 0.03 to 0.125 μg/ml for the five susceptible strains. The MICs for the 15-membered macrolides and lincosamides were higher than 64 μg/ml for the resistant strain while only 0.06 to 0.5 μg/ml for the susceptible strains. *Mycoplasma hyopneumoniae* strains are intrinsically resistant to the 14-membered macrolides due to a G2057A transition (*E. coli* numbering) in their 23S rDNA. Therefore, high MICs were observed for all strains, although the MICs for the resistant strain were clearly increased. An additional, acquired A2058G point mutation was found in the 23S rRNA gene of the resistant strain. No differences linked to resistance were found in the ribosomal proteins L4 and L22. The present study showed that 23S rRNA mutations resulting in resistance to macrolides and lincosamides as described in other *Mycoplasma* spp. also occur under field conditions in *M. hyopneumoniae*.

INTRODUCTION

MYCOPLASMAS ARE THE SMALLEST FREE-LIVING ORGANISMS, carrying no cell wall. As a consequence, they are naturally resistant to antibiotics interfering with cell wall synthesis. Additionally, a number of reports indicate a decrease in susceptibility of mycoplasmas against widely used antimicrobial agents, including the macrolides, lincosamides, and streptogramins (MLS).[2,12,15,25,35,37] MLS antibiotics have overlapping binding sites on the 23S rRNA and, hence, related antimicrobial activities. By binding to domain V of the 23S rRNA, they inhibit protein synthesis by means of blocking the path through which nascent peptides exit the ribosome.[28,33] Additional studies have mapped the recognition site of the 14-membered macrolide erythromycin and its derived ketolides to domain II and IV of the 23S rRNA as well.[4,29,36]

Bacterial species containing only one or two copies of rRNA genes, like all *Mycoplasma* species, tend to use mutations at bases 2,057–2,059 of the 23S rRNA as a way of acquiring resistance.[12,16] Some mycoplasmas, like *M. hyopneumoniae*, are intrinsically resistant to 14-membered macrolides due to a G2057A transition in their 23S rDNA.[12,20,30] Additional resistance to MLS antibiotics due to mutations at positions 2,609–2,611 has been observed for several bacterial species.[6,14,25] A mutation at position 2,062 was linked to resistance against josamycin, a 16-membered macrolide, in an *in vitro*-selected *Mycoplasma hominis* strain.[11,16] For a number of other bacteria, mutations in the L4 and L22 binding proteins were linked to MLS resistance.[6,8,9,19,26,27,29,31] Nonetheless, acquired resistance to MLS in *Mycoplasma* species is rarely documented or is induced *in vitro* rather than observed in field isolates.

The aim of this study was to characterize fully both phenotypically and genetically the *in vivo* acquired resistance to macrolides and lincosamides in a *M. hyopneumoniae* field isolate.

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TABLE 1. SELECTED PRIMERS USED FOR THE AMPLIFICATION AND SEQUENCING OF THE 23S, L4, AND L22 GENES OF THE *M. hyopneumoniae* J REFERENCE AND FIELD STRAINS

Primer name	*Primer sequence (5′→3′)*	*Number of cycles (cycle conditions)*[a]
L4 FOR	AGCATTCAAAGTCAGAAAAC	25 (30 sec at 94°C; 30 sec at 47.6°C; and 1 min at 72°C)
L4 REV	GATTCTCTTCTCCAAATTAG	
L22 FOR	AGCAGTCGCTTCACTCAAAA	25 (30 sec at 94°C; 30 sec at 52.5°C; and 1 min at 72°C)
L22 REV	ACCTCTTTTTCTTGCGCTAA	
23S FOR	GGTAGCGAAATTCCTTGTCA	25 (30 sec at 94°C; 30 sec at 55.2°C; and 1 min at 72°C)
23S REV	GAGCAGCTCTCATCAATATTCC	

[a]All PCRs, unless stated otherwise, were performed using 2 U of recombinant *Taq* polymerase, 5 μl of 10× PCR buffer, 75 nmol $MgCl_2$, 10 nmol of each dNTP, and 10 pmol of both forward and reverse primer.

MATERIALS AND METHODS

Media, bacterial isolates, and antibiotics

Isolates and media. The F2, F5, F6, F18, and F19 strains were all Belgian *M. hyopneumoniae* field strains isolated from lung tissue of fattening pigs collected at slaughterhouses. The J strain (ATCC 25934) was used as a control strain. The Friis' broth[10] without added antibiotics was used to grow the *M. hyopneumoniae* strains.

Antibiotics. Erythromycin, tylosin, and clindamycin were obtained from Sigma (UK). The other antibiotics were obtained from the drug's manufacturer as reference powder: lincomycin and azithromycin were kindly supplied by Pfizer (NY), clarithromycin by Abott (IL).

MIC test by a macro broth dilution technique. Antibiotic stock solutions of 1,000 μg/ml were freshly prepared the day of use according to guidelines described by the National Committee for Clinical Laboratory Standards (NCCLS).[22] Clarithromycin, erythromycin, and azithromycin were dissolved in a minimum amount of ethanol and further diluted in water. All other antibiotics were directly dissolved in water. For each antimicrobial, two-fold dilutions ranging from 0.015 μg/ml to 64 μg/ml were prepared and each strain was tested twice. Broths were inoculated with the *M. hyopneumoniae* strains, resulting in approximately 10^5 color-changing units (CCU) per ml in a final volume of 2 ml. An additional tube contained only medium and was used as a negative control, while a second tube without antibiotics served as a positive control. The MIC was defined as the lowest concentration of antimicrobial agent in which no growth of *M. hyopneumoniae* was observed. Growth was detected by a color change of the Phenol Red indicator from red to yellow due to glucose fermentation. The test was ended when no more color change in the tubes was visible during 2 consecutive days.

DNA sequencing of the L4- and L22-genes and domain V of the 23S rDNA

To sequence the L4 and L22 proteins, primers were designed based on the *M. hyopneumoniae* genome sequence of reference strain 232.[21] Primers for the amplification of domain V of the 23S rDNA were selected based on the *M. hyopneumoniae* ATCC 27719 strain (GenBank accession number X68421). All primers and reaction conditions used are listed in Table 1. After purification of the PCR product on Microcon 100 columns (Millipore, MA), both strands were sequenced on a CEQ8000

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                          |2040       |2050       |2060       |2070       |2080       |2090       |2100
E. coli[a]                GTACCCGCGG CAAGACGGAA AGACCCCGTG AACCTTTACT ATAGCTTGAC ACTGAACATT GAGCCTTGATG
M. hyopneumoniae[b]       TTACCCGCAT CAAGACGAAA AGACCCCGTG GAGCTTTACT ATAACTTCGT ATTGAGAATT GGTTTATTATG
M. hyopneumoniae F19      TTACCCGCAT CAAGACGAGA AGACCCCGTG GAGCTTTACT ATAACTTCGT ATTGAGAATT GGTTTATTATG
M. hominis                AGACCCGCAT CTAGACGAAA AGACCCCGTG GAGCTTTACT ATAACTTCAT ATTGGAGTTT GATTTAACATG
M. fermentans             GTACCCGCAT CAAGACGAAA AGACCCCATG GAGCTTTACT ACAGTTTCGT ATTGGAACTT GGTCTAACATG
M. genitalium             TTAGGCGCAA CGGGACGGAA AGACCCCGTG AAGCTTTACT GTAGCTTAAT ATTGATCAAA ACACCACCATG
M. pneumoniae             TTAGGCGCAA CGGGACGGAA AGACCCCGTG AAGCTTTACT GTAGCTTAAT ATTGATCAGG ACATTATCATG
M. gallisepticum          TTAGGCGCAA CGGGACGGAA AGACCCCATG AAGCTTTACT GTAACTTAAT ATTGGGCAGA GTTTAGACATA
M. penetrans              TTAGGTGCGG TTAGACAAAA AGACCCCATG AAGCTTTACT GTAGCTTAAT ATTGGAAAAA TTTATTTCATT
M. flocculare             TTACCCGCAT CAAGACGAAA AGACCCCGTG GACGTTTACT ATAACTTCGT ATTGAGAATT GGTTTATTATG
M. hyorhinis              CGAACCGTAG TACGCTAAAA AGTTGCCCGGA TGACTTGTGG ATAGC----- GGTGAAATTC CAATCGA-ACC
M. pulmonis[c]            CGAACCGTAG TACGCTGAAA AGTTGCCCGGA TGACTTGTGA ATAGC----- GGTGAAATTC CAATCGA-ACC
                            *   *       *     * **  ***         **        *          **              *
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[a]Sequences were extracted from Genbank. Accession numbers are: *Escherichia coli*, EC0278710; *M. hominis*, AF443617; *M. fermentans*, AF422142; *M. genitalium*, U39634; *M. pneumoniae*, AE000007; *M. gallisepticum*, AE016968; *M. penetrans*, AP004174; *M. flocculare*, MYC16SR; *M. hyorhinis*, AF121891; *M. pulmonis*, AL445565.

[b]No differences in the 23S DNA of strain F2, F5, F6, F18, F23, and J were observed.

[c]Resistance due to A2062 point mutations have been described elsewhere.[10]

FIG. 1. Multiple sequence alignment of the 23S rDNA of different *Mycoplasma* spp. compared to *E. coli*. Nucleotides related to resistance to macrolides are presented in bold. The G2058A transition of the resistant *M. hyopneumoniae* F19 strain is underlined as well.

sequencer (Beckmann, UK) according to the manufacturer's instructions. The sequences were aligned for further analysis using Clustal W (V1.82). The sequences of domain V of the 23S rDNA of the *M. hyopneumoniae* strains were compared with sequences of *E. coli* and other *Mycoplasma* species extracted from GenBank (Fig. 1).

RESULTS

Because the isolates grew at different rates, not all MICs were read on the same day. The J reference strain grew faster than the other isolates, and the MICs of the antibiotics were reached after approximately 5 days, whereas it took up to 1 week for the other strains. The MICs for the isolates are presented in Table 2. For each isolate, the MIC values of the replicates were equal to or differed from each other by only one dilution. The MICs for erythromycin and clarithromycin ranged from 8 to 32 μg/ml for all strains tested, except for the resistant F19 isolate. For this strain, the MIC values exceeded the highest concentration (64 μg/ml) tested. All isolates, except the F19 strain, were also susceptible to the other antibiotics tested. The MICs ranged from 0.06 to 0.25 μg/ml for the 15-membered macrolide, azithromycin; from 0.03 to 0.125 μg/ml for the 16-membered macrolide, tylosin; and from 0.125 to 0.5 μg/ml for the lincosamides, lincomycin, and clindamycin. The MICs for the resistant isolate (F19) also exceeded the highest concentration of 64 μg/ml for these antibiotics, with the exception of tylosin (MICs ranging from 8 to 16 μg/ml).

Sequence analysis of the 23S rDNA, as shown in Fig. 1, demonstrates the intrinsic resistance of certain *Mycoplasma* spp. to 14-membered macrolides due to a G2057A transition. An acquired A2058G transition was found exclusively in the resistant *M. hyopneumoniae* strain. Only a small number of differences were present in the L22 proteins (>97% identity at DNA level). Protein L4 proved very conserved (>99% identical at DNA level) as no amino acid substitutions were found between any of the strains (data not shown).

DISCUSSION

Because *M. hyopneumoniae* strains are difficult to grow on agar plates and colonies are hard to detect, a serial broth dilution technique using Friis' medium was chosen to test for antibiotic resistance. This technique, based on an earlier report of Ter Laak *et al*.,[34] was performed in duplicate and proved to be reproducible.

The J reference strain seemed better adapted to the Friis' medium and grew faster than the other isolates. This may explain the higher MICs, up to one dilution, for this strain. Nevertheless, all individual MICs were very similar for the susceptible strains and clearly differed from those of the resistant F19 strain. Moreover, MICs for the sensitive strains were consistent with previous reports.[18,32,34]

Although *M. hyopneumoniae* strains are naturally resistant to 14-membered macrolides due to a G2057A transition of the 23S gene,[36] even higher MICs were observed for the resistant strain. This increased resistance may well be explained by the observed A2058G transition because footprinting patterns examining the drug binding sites in other bacteria identified these specific nucleotides.[7,17] Hence, a lower affinity of the drugs to the 23S rRNA, due to the acquired mutation, results in a decreased antimicrobial activity. Apart from this increased insensitivity to erythromycin and clarithromycin, the F19 field isolate proved also resistant to all other antibiotics tested. This is in agreement with earlier reports in other bacterial species where mutations at position 2,058 led to high MLS resistant strains.[7,36] This A2058G transition may even be the most frequently observed substitution *in vivo* in association with MLS resistance[36] and was also found for clinical isolates of *M. pneumoniae*,[24] although only limited data exist for *in vivo*-acquired MLS resistance in *Mycoplasma* species.

Recently, resistance to macrolides and lincosamides has been linked to modifications in the L4 and L22 rRNA-binding proteins,[13,23] especially in pneumococcal strains.[6,9,13,19,23,27,31] In the present studies, none of the variations in these proteins was uniquely present in the resistant *M. hyopneumoniae* field isolate F19, and it is therefore unlikely that they are associated with acquired resistance. This is in agreement with an earlier report of resistant clinical strains of *M. hominis* and *M. fermentans*.[25]

In *in vitro* experiments, resistance to tylosin was obtained in *M. hyopneumoniae* strains after only seven or fewer passages in selective media,[15] indicating that acquired resistance due to mutations of the 23S RNA may occur quite fast for bacteria like *M. hyopneumoniae*, which only possess one copy of the rRNA operon.[30] The relation between the use of macrolides in pig rearing and the occurrence of acquired resistance has been indirectly demonstrated for *M. hyosynoviae* and other porcine

TABLE 2. MICs (μG/ML) OF MLS-ANTIMICROBIAL AGENTS FOR BELGIAN *M. hyopneumoniae* FIELD ISOLATES AND THE *M. hyopneumoniae* J REFERENCE STRAIN OBTAINED BY THE BROTH DILUTION TEST CARRIED OUT IN TWO-FOLD

	MIC (μg/ml)					
Strain	*Azithromycin*	*Tylosin*	*Erythromycin*	*Clarithromycin*	*Clindamycin*	*Lincomycin*
F2	0.125[a]	0.03125	8	16	0.0625	0.25
F5	0.125–0.25	0.03125	8	16	0.125–0.25	0.25
F6	0.0625–0.125	0.03125	16	32	0.125	0.125
F18	0.0625	0.0625	32	32	0.125–0.25	0.125
F19	>64	8–16	>64	>64	>64	>64
J	0.25	0.125	32	32	0.5	0.5

[a]One value means that no difference between the repeated tests was observed.

bacteria.[1,2,5] In contrast, the prevalence of acquired resistance to macrolides for *M. hyopneumoniae* in the field is most likely low.[18,37] It is possible that resistant strains do not spread easily and that occurrence of resistance remains localized in an area or even within a herd. Indeed, earlier RAPD data[3] and our own PFGE results (unpublished) show an enormous diversity among isolates from different farms, suggesting that clones do not readily spread in the environment, although further research on this issue is needed. Because the type of resistance described above is not encoded on a mobile element, and therefore not transferable between different strains, the genetic stability of the mutation may also be an important factor. Although the A2058G transition has distinct advantages over the wild-type in the presence of macrolides, the situation may be different in the absence of the drugs.[36] In any event, the emergence of this resistance asks for a continuous monitoring, because it may have important therapeutic implications in the treatment of mycoplasma infections.

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