Induction of macrolide resistance in *Mycoplasma gallisepticum* in vitro and its resistance-related mutations within domain V of 23S rRNA

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Abstract

Antibiotic-resistant mutants of *Mycoplasma gallisepticum* were selected in vitro from the susceptible strains S6 and BG44T by serial passages in stepwise concentrations of erythromycin, tylosin, or tilmicosin. High resistance to erythromycin or tilmicosin developed readily, whereas resistance to tylosin developed only after greater numbers of passages. Three mutants selected by each selector antibiotic were cloned and detected, and all cloned mutants exhibited cross-resistance to the three selector antibiotics as well as to lincomycin. Portions of the genes encoding domain V of 23S rRNA of the cloned mutants were amplified by PCR, and their nucleotide sequences were compared to those of the susceptible parent strains. Five of the six mutants selected by erythromycin harbored an A2058G (*Escherichia coli* numbering) mutation in one of the two 23S rRNA. One of the six mutants selected by erythromycin harbored a G2057A mutation and an A2059G mutation in the other 23S rRNA. In tilmicosin-selected mutants, two mutations, A2058G and A2503U, occurred in one of the two 23S rRNA. No mutation was detected in the two 23S rRNA of tylosin-selected mutants with low-level resistance. Mutations at homologous locations in the 23S rRNA of other macrolide-resistant bacteria indicate that the phenotype of macrolide resistance occurring in *M. gallisepticum* is strongly associated with point mutations in domain V of 23S rRNA.

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Keywords: *Mycoplasma gallisepticum*; Antibiotic resistance; Macrolide; 23S rRNA mutation

1. Introduction

*Mycoplasma gallisepticum* is responsible for chronic respiratory diseases (CRD) in chickens as well as sinusitis in turkeys [1]. The worldwide poultry industry incurs significant economic losses from insufficient growth, death and carcass disposal of the infected birds. Various measures have been implemented in many countries in the hope of eradicating the disease, but the results were far from satisfactory. Because of this, chemotherapeutic approaches are often necessary to minimize *M. gallisepticum* transmission in the case of outbreaks.

Macrolides are a common drug choice for treatment of *M. gallisepticum* infection. However, resistance readily
develops when these drugs are used frequently [2–7]. Tilmicosin (20-deoxo-20-(3,5-dimethylpiperidin-1-yl)desmycosin), a new chemically modified macrolide antibiotic firstly developed by Elanco Animal Health (a division of Eli Lilly and Company USA) in 1980s, is synthesised from tylosin by sequential hydrolysis of mycarose and reductive amination of the aldehyde in demycarosyltylosin (desmycosin) with 3,5-dimethylpiperidine [8]. Tilmicosin has an antibacterial spectrum similar to tylosin, but with enhanced activity against gram-negative bacteria such as Pasteurella spp. and Actinobacillus spp. [8–10]. Therefore, it is currently approved exclusively for veterinary use to treat and prevent pneumonia in cattle and pigs associated with Pasteurella haemolytica, Pasteurella multocida, Actinobacillus pleuropneumoniae and other bacteria found sensitive to this compound [11,12]. Tilmicosin is also active in vitro against mycoplasma including Mycoplasma gallisepticum [8,9,13]. Several studies [13–15] have shown that tilmicosin has good efficacy against experimental infection of chickens and turkeys with Mycoplasma gallisepticum. These reports suggest that tilmicosin is a promising drug in the control of Mycoplasma gallisepticum infection.

The development of effective therapeutic strategies depends upon an accurate and detailed understanding of the antibiotic resistance mechanisms. Only a few studies have reported in vitro emergence of resistance to macrolides in Mycoplasma gallisepticum [16–18]. To our knowledge, no studies have examined tilmicosin resistance, and there is relatively little data in the literature concerning the genetic basis for macrolide resistance in Mycoplasma gallisepticum. The Mycoplasma gallisepticum genome only contains two rRNA operons [19]. Vester and Douthwaite [20] reported that ribosomal RNA mutations underlie resistance to macrolides in bacteria possessing a low number of rRNA operons. The interaction sites for macrolides in bacteria are located primarily in the peptidyltransferase region within domain V of the 23S rRNA [21,22]. In this study we compared the potential of selection resistance in Mycoplasma gallisepticum to tilmicosin with those to erythromycin and tylosin, and attempted to establish a possible relationship between the resistant phenotype and the appearance of specific point mutations in domain V of the 23S rRNA.

2. Materials and methods

2.1. Bacterial strains, growth conditions, and antibiotics

Two strains of Mycoplasma gallisepticum, S6, a reference strain, and BG44T, a field strain obtained from flocks in Beijing, China, were used to select macrolide-resistant mutants. Prior to our study, these two strains were clone-purified once and were tested for identity by the growth-inhibition test [23] using antiserum prepared in rabbits to Mycoplasma gallisepticum. The cloned strains were inoculated at 37 °C in Mycoplasma broth for 5–7 days until a color change (pink to orange-yellow) occurred. After addition of 20% (v/v) of sterile glycerol, the cultures were aliquoted and stored at −70 °C. Mycoplasma broth (MB) was prepared according to the FM-4 formula [24] and supplemented with 15% swine serum, 0.025% thallium acetate, 0.002% phenol red and 10,000 IU/ml penicillin G, respectively. Mycoplasma agar (MA) was similar to MB but contained 1% Noble agar (Difco). The following antibiotics were used: tilmicosin (Jixing Pharmaceutical and Chemical Ltd, Shan-dong Province, China), erythromycin, tylosin (both obtained from the China Institute of Veterinary Drug Control, Beijing, China), and lincomycin (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China).

2.2. Selection of macrolide-resistant Mycoplasma gallisepticum mutants

Macrolide-resistant mutants were selected as described by Reinhardt et al. [25]. Briefly, for each passage, 1 ml of MB medium, containing about 10^5 color changing units (CCU) inoculum, was set up either in antibiotic-free microtubes (Costar) or in microtubes containing one of seven antibiotic concentrations. These concentrations consisted of the minimum inhibitory concentration (MIC) of the antibiotic for the parent strain and three twofold increases above and below this value. The length of the incubation period of the cultures at 37 °C was coordinated with the color change (from pink to orange-yellow) of the antibiotic-free culture growth control. The cultures with the highest concentration of antibiotics and with identical color changes were then retained to establish the subsequent cultures. The highest antibiotic concentration that also allowed the growth of the culture in the previous passage was used, together with three twofold increases above and below this value. After reaching 128 µg/ml of selector antibiotic, or after the 10th passage, aliquots of the retained cultures were sub-cultured on MA plates to obtain cloned mutants for MIC determinations and genetic analyses.

2.3. MIC determinations

The MICs of each antibiotic for Mycoplasma gallisepticum parent strains and for the cloned mutants were determined by the broth dilution method in 96-well microtiter plates [26]. Briefly, each well of the microtiter plates contained stepwise twofold concentrations of an antibiotic and an inoculum of 10^5 CCU in 0.2 ml of MB medium. The microtiter plates were incubated at 37 °C and examined daily for 5–7 days. MIC is defined as the lowest concentration of antibiotic that prevent a color change.
in the medium at the time when the antibiotic-free growth control showed a color change. All MIC tests were repeated three times to confirm the results.

2.4. PCR amplification, cloning, and sequencing of the domain V of 23S rRNA genes

Restriction endonuclease digestion and Southern hybridization analysis were performed in order to separate the different copies of 23S rRNA genes in the *M. gallisepticum* genome [27]. Intact *M. gallisepticum* genomic DNA was extracted as previously described [28] and digested with restriction endonuclease *Bgl* II (Promega). Following electrophoresis in 0.8% agarose gels, restriction fragments were transferred to nylon membranes and hybridized to DIG-labeled probes according to the manufacturer’s instructions for the DIG labeling and detection kit (Roche). The probes were 919-bp DNA fragments including domain V of the 23S rRNA gene. They were generated by amplification from the genomic DNA of *M. gallisepticum* S6 with the primers 5’-GTGAAAGACGAGGGGACTGT-3’ and 5’-TCATCCGTTCTCTGACT-3’, whose design was based on the sequences of *M. gallisepticum* strain AS969 (GenBank accession no. AF036708). The fragment bands, whose locations corresponded to hybridization bands on agarose gels, were excised and used as PCR templates. PCR was performed with the aforementioned primers and *pyrobest* DNA polymerase (Takara). Thirty-two cycles of polymerization reactions (1 min at 94°C, 70 s at 47°C, and 110 s at 72°C) resulted in the expected fragment, which was purified by electrophoresis in 1% agarose and extracted with QIAquick Gel Extraction Kit (QIAGEN). Fragments were ligated to pGEM-T easy vectors (Promega) and introduced into *Escherichia coli* DH5α, and then determined with the ABI PRISM Big Dye Primer Cycle Sequencing Ready Reaction Kit and the ABI 377 DNA auto-sequencing machine (Applied Biosystems) by using T7 and SP6 sequence primers (Perkin–Elmer). The sequences from the mutants were aligned with the corresponding sequences from the parent strains and *E. coli* (GenBank accession no. J01695) with the software DNAMAN (Lynnon Biosoft).

3. Results

The development of the antibiotic concentration used during the induction of macrolide resistance in *M. gallisepticum* is outlined in Fig. 1. Regardless of the *M. gallisepticum* strain used for the selection, resistance to erythromycin developed easily and quickly. After 5–6 passages, mutants tolerated at least 128 µg/ml of erythromycin. As for tilmicosin, the increase of concentrations was slow for the first 4 passages, but then sped up and reached 128 µg/ml after 7–8 passages. However, the rise in concentrations of tylosin was slow and fluctuating. Even after 10 passages the mutants could not resist 0.125 µg/ml of tylosin.

For each selector antibiotic, three clones, derived from both *M. gallisepticum* S6 and BG44T, were obtained after the selection. The resistance phenotype remained stable in all the cloned mutants after three consecutive sub-cultures in the antibiotic-free medium. MIC results for *M. gallisepticum* S6, BG44T and the cloned mutants are shown in Table 1.

The parent strains were susceptible to all the selector antibiotics as well as to lincomycin. Regardless of whether *M. gallisepticum*, S6 or BG44T was used for the selection, the mutants obtained after 5–6 passages in the presence of erythromycin were highly resistant to erythromycin (MICs > 256 µg/ml), and were also cross-resistant to tylosin (MICs = 1–2 µg/ml), tilmicosin (MICs = 4–8 µg/ml) and lincomycin (MICs > 512 µg/ml). In particular, one of three BG44T-derived mutants showed a higher resistance to tylosin (MICs = 64 µg/ml) and tilmicosin (MICs = 64–128 µg/ml) than did the other two. Likewise, the MIC results indicate that the mutants obtained after 7–8 cycles of tilmicosin exposure were significantly resistant to tilmicosin, and were also highly cross-resistant to erythromycin, tylosin, and lincomycin. In contrast, sub-culturing in the presence of tilmicosin did not select highly resistant *M. gallisepticum* mutants. After 10 serial passages, the MICs of tilmicosin for the mutants were only 16 and 32 times the MICs of tilmicosin for S6 and BG44T, respectively.

To investigate the role of domain V of the 23S rRNA encoded in macrolide resistance, the two copies of 23S rRNA genes in *M. gallisepticum* were differentiated by restriction endonuclease digestion and Southern hybridization analysis. As expected, two distinct fragment bands (corresponding to sizes of 3.1 and 3.8 kb in digests of S6 genomic DNA) hybridized with the probes responding to sizes of 3.1 and 3.8 kb in digests of S6 genomic DNA. The two clones derived from *M. gallisepticum* S6 and BG44T, were also differentially resistant to antibiotics as well as to lincomycin. Regardless of whether *M. gallisepticum*, S6 or BG44T was used for the selection, the mutants obtained after 5–6 passages in the presence of erythromycin were highly resistant to erythromycin (MICs > 256 µg/ml), and were also cross-resistant to tylosin (MICs = 1–2 µg/ml), tilmicosin (MICs = 4–8 µg/ml) and lincomycin (MICs > 512 µg/ml). The sequences encoding domain V of 23S rRNA from the mutants were aligned with the corresponding sequences from the parent strains and *E. coli* (GenBank accession no. J01695) with the software DNAMAN (Lynnon Biosoft).
position 2058 in one of the two 23S rRNA copies. However, one mutants derived from *M. gallisepticum* BG44T did not display the A2058G mutation in the same 23S rRNA, but instead harbored two mutations, G2057A and A2059G, in the other 23S rRNA copy. Regardless of the *M. gallisepticum* strain used for the selection, no base mutation in domain V of the two 23S rRNA copies was identified in the cloned mutants selected by tylosin.

### Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (µg/ml)</th>
<th>Mutations in domain V of 23S rRNA copy number 1</th>
<th>Mutations in domain V of 23S rRNA copy number 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EM</td>
<td>TL</td>
<td>TM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23S rRNA copy number 1</td>
<td>23S rRNA copy number 2</td>
</tr>
<tr>
<td>S6</td>
<td>0.032</td>
<td>0.016</td>
<td>0.008</td>
</tr>
<tr>
<td>S6-EM5</td>
<td>256 ≥ 512</td>
<td>1–2</td>
<td>4–8</td>
</tr>
<tr>
<td>S6-TL10</td>
<td>1–2</td>
<td>0.125–0.25</td>
<td>0.125–0.25</td>
</tr>
<tr>
<td>S6-TM7</td>
<td>≥ 512</td>
<td>16–32</td>
<td>256</td>
</tr>
<tr>
<td>BG44T</td>
<td>0.063</td>
<td>0.008</td>
<td>0.004</td>
</tr>
<tr>
<td>BG44T-EM6</td>
<td>256</td>
<td>2–64</td>
<td>8–128</td>
</tr>
<tr>
<td>BG44T-TL10</td>
<td>1–2</td>
<td>0.125–0.25</td>
<td>0.125–0.25</td>
</tr>
<tr>
<td>BG44T-TM8</td>
<td>≥ 512</td>
<td>16–32</td>
<td>256–512</td>
</tr>
</tbody>
</table>

–, No mutation was observed.

* M. gallisepticum S6 and BG44T are parent strains. The cloned mutants are designated by the parent strain, the selector antibiotic and the passage number.

* EM, Erythromycin; TL, Tylosin; TM, Tilmicosin; LM, Lincomycin.

* Escheria coli numbering.

* The number of clones that mutation occurred in them.
after 10 passages. Two mutations, A2058G and A2503U occurred in domain V of one of the two 23S rRNA copies in the cloned mutants selected by tilmicosin.

4. Discussion

In the present study, high-level resistance to erythromycin developed in M. gallisepticum after 5–6 passages. This result is in general agreement with previous findings [16,17]. Unexpectedly, however, all of the erythromycin-resistant mutants obtained in our study exhibited cross-resistance to tylosin, tilmicosin, and lincomycin. This phenomenon is notable and requires further study. In the poultry industry, erythromycin is used widely against M. gallisepticum. The cross-resistant mutants selected by erythromycin will influence the efficacy of tylosin and tilmicosin in treatments.

M. gallisepticum resistance to tylosin developed very slowly, in that the mutants had only a low-level resistance to tylosin and other related antibiotics even after 10 passages. These results are in contrast with Gautier-Bouchardon et al.’s findings [17] that high concentrations of tylosin were reached rapidly (2–6 passages) during the induction of tylosin resistance in M. gallisepticum. These results also differ from the report of Zanella et al. [16], who showed that resistance to tylosin developed slowly (8–11 passages) although the mutants exhibited high resistance to tylosin. However, our results are in general agreement with the results obtained by Takahashi et al. [18], who reported that acquisition of resistance in M. gallisepticum against tylosin in vitro was difficult and slow, and maximum growth allowance concentrations (MAC) of the 10th sub-culture only increased 8 times for both strains as compared to those of the primary sub-cultures.

Tilmicosin is a tylosin-related macrolide prepared by chemical modification of desmycosin [8]. Although for the two parental strains of M. gallisepticum the MIC of tilmicosin is nearly equal to that of tylosin, resistant mutants were obtained more readily and more quickly with tilmicosin than with tylosin. Results of cross-resistance trials showed that tilmicosin and tylosin share a complete cross-resistance in M. gallisepticum. Moreover, the tilmicosin-selected mutants were also resistant to erythromycin and lincomycin. To our knowledge, this is the first description of an in vitro selection of tilmicosin resistance in M. gallisepticum.

Five of the six M. gallisepticum mutants selected by erythromycin showed cross-resistance to tylosin, tilmicosin and lincomycin, and also harbored an A2058G mutation in domain V of 23S rRNA. The same mutation in other organisms is known to result in resistance to erythromycin and related antibiotics. Erythromycin-resistant strains of M. pneumoniae isolated in vitro [29] or in vivo [30] have been found to harbor A2058G. Thus, the A2058G mutation in domain V of 23S rRNA is linked to the observed resistance phenotype in M. gallisepticum.

Notably, one of the erythromycin-selected mutants (derived from BG44T) harbored two mutations, G2057A and A2059G in domain V of 23S rRNA and not the A2058G mutation, although it exhibited the same resistant phenotype as other erythromycin-selected mutants. This mutation pattern had not been observed previously in mycoplasma species. A natural G2057A transition was found in M. hominis, M. fermentans [31], M. pulmonis [32], M. hyopneumoniae [33], and
with their C5 amino sugar in a similar orientation; however, 14- and 16-membered macrolides adopt different conformations, thereby enabling the latter compounds to avoid a steric clash with 2058G [38]. Furthermore, tylosin has a mycaminose-mycarose disaccharide at the C5 position, which extends back up the tunnel towards the peptidyl-transferase center. This feature enables additional interactions, most notably at G2505 [39] and U2506 [40] that are outside the reach of the erythromycin. Tilmicosin is a semi-synthetic derivative of tylosin with a monosaccharide at the C5 position in the lactone ring instead of a mycaminose-mycarose disaccharide, and a 3,5-dimethylpiperidine at C6 position [8]. Without a mycarose sugar, the inability of tilmicosin to interact with G2505 may be analogous to the interaction with desmycin [40]. If true, this implies that a single mutation at 2058 or 2059 will have a more noticeable effect on tilmicosin resistance.

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