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Acquired resistance to the 16-membered macrolides tylosin and tilmicosin by *Mycoplasma bovis*

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ABSTRACT

The molecular mechanism of acquired resistance to the 16-membered macrolides tylosin (Ty) and tilmicosin (Tm) was investigated in *Mycoplasma bovis* field isolates. Sequence analysis of domains II and V of the two 23S rRNA alleles and ribosomal proteins L4 and L22 was performed on 54 *M. bovis* isolates showing different minimal inhibitory concentrations (MIC). The presence of any one of the point mutations G748A, C752T, A2058G, A2059G or A2059C (*Escherichia coli* numbering) in one or both alleles of the 23S rRNAs was correlated with decreased susceptibility to Ty (8–1024 μg/ml) and to Tm (32 to >256 μg/ml) in 27/27 and 27/31 *M. bovis* isolates, respectively. Although a single mutation in domain II or V could be sufficient to cause decreased susceptibility to Ty, our data imply that a combination of mutations in two domains is necessary to achieve higher MICs (≥128 μg/ml). The influence of a combination of mutations in two domains II and V on enhancement of resistance to Tm was less clear. In addition, the amino acid (aa) substitution L22-Q90H was found in 24/32 representative *M. bovis* isolates with different MICs, but no correlation with decreased susceptibility to Ty or Tm was identified. Multiple aa substitutions were also identified in the L4 protein, including at positions 185–186 (positions 64 and 65 in *E. coli*) which are adjacent to the macrolide-binding site. This is the first description of the molecular mechanism of acquired resistance to the 16-membered macrolides in *M. bovis*.

1. Introduction

Mycoplasma bovis causes a range of clinical conditions including pneumonia, mastitis, arthritis, otitis and reproductive disorders. Moreover, it plays a significant role in bovine respiratory disease (BRD) which is the most common disease affecting housed cattle worldwide and is a major impediment to animal production, leading to substantial economic losses (Nicholas et al., 2009). Since no effective vaccine is commercially available, antibiotic treatment is the main method for attempting to control *M. bovis* infections. However, evidence from *in vitro* studies shows that isolates of *M. bovis* circulating in Europe and Israel have high minimum inhibitory concentrations (MIC) for many of the commercially available antibiotics (Ayling et al., 2000; Gerchman et al., 2009; Thomas et al., 2003).

The 16-membered macrolides tylosin (Ty) and tilmicosin (Tm) are widely used for prophylaxis and therapy in veterinary medicine including cattle. However, previous

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studies in our laboratories and elsewhere have shown significant resistance by *M. bovis* field isolates to these antibiotics in recent years (Ayling et al., 2000; Gerchman et al., 2009; Rosenbusch et al., 2005; Thomas et al., 2003). Acquired resistance to macrolides can arise *via* a number of different mechanisms including enzymatic inactivation of macrolides, increased macrolide efflux, methylation and alteration of the macrolide-binding site (Leclercq, 2002). It has been shown previously that point mutations at the macrolide-binding site in domains II and/or V of the 23S rRNA gene/s as well as in the *rplD* and *rplV* genes that encode ribosomal proteins L4 and L22, respectively, are the main mechanisms conferring resistance to macrolides in *Mycoplasma* species (Bebear et al., 2011; Gerchman et al., 2011; Kobayashi et al., 2005; Shimada et al., 2011; Stakenborg et al., 2005; Xiao et al., 2011). However, thus far, nothing is known about the mechanism of acquired resistance to macrolides in *M. bovis*. Therefore, the aim of this study was to characterize the *in vivo* acquired resistance to 16-member macrolides Ty and Tm in *M. bovis* field isolates.

2. Materials and methods

2.1. M. bovis field isolates and culture media

A total of 54 *M. bovis* field isolates were analyzed in this study. These included 32 isolates for which geographic origin, clinical condition, and susceptibility profiles have been previously described (Gerchman et al., 2009) and 22 newly-analyzed *M. bovis* isolated in Israel from local (14) cattle and cattle imported from Hungary (2) or Lithuania (1); two were isolated in Germany and three in the UK (Table 1). The reference type strain *M. bovis* PG45 was obtained from the Animal Health and Veterinary Laboratories Agency, Mycoplasma Group, Surrey, UK.

All isolates were propagated at 37 °C in standard *M. bovis* broth medium (Rosengarten et al., 1994) adjusted to pH 7.8, and later supplemented with 0.5% (w/v) sodium pyruvate and 0.005% (w/v) phenol red. Isolates of *M. bovis* were identified by direct immunofluorescence (IMF) of colonies using species-specific conjugated antiserum.

2.2. Antimicrobial agents and susceptibility testing

Susceptibility of *M. bovis* isolates to Ty (98% active substance), and Tm (91%) (Eli Lilly, Indianapolis, IN, USA) was tested by the microbroth dilution test as previously described (Gerchman et al., 2009) following the guidelines recommended by Hannan (2000). Two-fold dilutions of antibiotics from 0.5 to 128 μg/ml were tested. For *M. bovis* isolates with high MIC values for the macrolides in the preliminary test and those with previously published MIC >128 μg/ml (Gerchman et al., 2009), an additional round of testing with Ty and Tm in the range of 16–1024 μg/ml or 8–256 μg/ml, respectively, was performed. Due to the poor solubility of Tm, the maximum MIC that could be determined for this antibiotic was 256 μg/ml. The microbroth procedure was repeated independently three times for the reference strain PG45 and selected representative isolates, with the same results within a single twofold dilution (data not shown). MIC values for Ty and Tm in reference strain PG45 were 0.5 μg/ml for both antibiotic agents and were consistent with the previously published values for this strain (Hannan, 2000).

2.3. PCR amplification and sequence analysis of domains II and V of the 23S rRNA genes (rrl3 and rrl4), rplD and rplV genes

M. bovis genomic DNA was extracted from 20 ml of logarithmic-phase broth cultures using the DNeasy Blood

Table 1
Newly-analyzed *M. bovis* isolates.

N	Isolate	Year of isolation	Country of isolation	Clinical condition
1	277/83	1983	Germany	Mastitis
2	100/91	1991	Germany	Pneumonia
3	1716	2007	Israel	Pneumonia
4	8692	2007	Israel	BRD
5	7028	2007	Israel	Pneumonia
6	6127	2007	Israel	Pneumonia
7	268B07	2007	UK	Arthritis
8	7239	2008	Israel	Pneumonia
9	6656	2008	Israel	Pneumonia
10	7227	2008	Lithuania	Pneumonia
11	6512	2008	Israel	BRD
12	4426	2008	Israel	Pneumonia
13	5028	2008	Israel	Pneumonia
14	2600	2008	Hungary	Pneumonia
15	5936[a]	2008	Israel	Pneumonia
16	6866	2008	Hungary	Pneumonia
17	869[a]	2008	Israel	Pneumonia
18	5630	2008	Israel	Pneumonia
19	742B08	2008	UK	Mastitis
20	393B08	2008	UK	Mastitis
21	88127	2010	Israel	BRD
22	72242	2010	Israel	Pneumonia

[a] *M. bovis* isolates isolated on the same farm.