The Resistance Mechanism of *Mycoplasma bovis* From Yaks in Tibet to Fluoroquinolones and Aminoglycosides

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*Mycoplasma bovis* (*M. bovis*) is one of the important pathogens for yaks. Aminoglycosides and fluoroquinolones are frequently used medications for the treatment of *M. bovis*. Drug-resistant strains were inevitable with the abuse of antibiotics. The resistance of *M. bovis* to aminoglycosides was related to the base mutations in drug target genes. Amino acid mutations at the quinolone resistance-determining region (QRDR) in gyrA, gyrB, parC, and parE conferred resistance to fluoroquinolones. In order to investigate the resistance mechanism of *M. bovis* from yaks in Tibet to aminoglycosides and fluoroquinolones, six frequently used antibiotics and ten clinical *M. bovis* strains were administered for a drug sensitivity test for *in vitro*-induced highly resistant strains, a drug stable-resistance test, cross-resistance test, and analysis of target gene mutations. The results showed that the clinical strains of *M. bovis* from yaks in Tibet had varying degrees of resistance to fluoroquinolones and aminoglycosides. The mechanism of resistance to fluoroquinolones and aminoglycosides was identified preliminarily for *M. bovis* from yaks: the single-site base mutation mediated the resistance of *M. bovis* from yaks and both base mutations led to highly resistant strains (aminoglycosides: rrs3 and rrs4; fluoroquinolones: gyrA and parC). The active efflux system results of *M. bovis* showed that there was no active efflux system based on fluoroquinolones and aminoglycosides expressed in *M. bovis* from yaks. The research could provide a reference for clinical treatment of *M. bovis*.

**Keywords:** fluoroquinolones, resistance mechanism, yak, *Mycoplasma bovis*, aminoglycosides

**INTRODUCTION**

*Mycoplasma bovis* (*M. bovis*) is one of the important pathogens that causes bovine disease syndromes such as pneumonia, mastitis, keratoconjunctivitis, arthritis, genital tract inflammation, miscarriage, and infertility (1). Sick cattle and the respiratory modes of transmission were the main sources and routes of infection (2). *Mycoplasma* was isolated firstly from mastitis milk by Hale et al. (3). It caused huge economic losses to the cattle industry (4).

In China, *M. bovis* was first isolated by Li (5) and proved to be the pathogen of respiratory diseases in cattle until 2008 (5–8). Antibiotics (aminoglycosides and fluoroquinolones) have been used to treat *M. bovis*. Previous research confirmed the resistance to macrolides and fluoroquinolones for *M. bovis* clinically isolated strains (9, 10). In our previous study, we found...
that the isolated strains were resistant to macrolides, aminoglycosides, and lincosamides (11). The research was imperative for drug resistance mechanisms (12).

Aminoglycosides and fluoroquinolones are broad-spectrum antibiotics against Gram-negative bacteria (13). The resistance of *M. bovis* to aminoglycosides is related to the base mutations in drug target genes (14). Previous research showed that there was a base mutation in 16S rRNA, but no base mutation was detected in S12 ribosomal protein (15–17). Amino acid mutations at the quinolone resistance-determining region (QRDR) in gyrA, gyrB, parC, and parE conferred resistance to fluoroquinolones (18). The mutation in the QRDR of gyrA contributed to nalidixic acid resistance (19). The amino acid mutation in parC was the leading cause of fluoroquinolones resistance in bacteria (20). The efflux pump mediated the resistance to aminoglycosides by AmrB, MexY, and AcrD genes and to fluoroquinolones by EmrAB. AcrAB. YdhE. AcrEF, and MdfA genes in resistance nodulation cell division (21, 22).

In our study, we performed antibiotic susceptibility testing, drug target gene mutation analysis, and developed a preliminary confirmation of an active drug efflux system on 10 isolates of *M. bovis* from Tibet yaks. It could provide references for the drug-resistant mechanism of *M. bovis*.

**MATERIALS AND METHODS**

**Strains**

Ten isolated strains of *M. bovis* from yaks in Tibet (Tibet-1~10) came from the Key Laboratory of Tibet Plateau Animal Disease Research Autonomous Region (11).

**Design and Synthesis of Primers**

The DNA of *M. bovis* was extracted by the boiling method (23). The primers were designed as described in a previous research paper (Table 1) (17, 18).

**Antibiotic Susceptibility Testing**

The minimum inhibitory concentration (MIC) of the 10 strains were detected by the microdilution method (17) against SPE (spectinomycin), GEN (gentamicin), KAN (kanamycin), ENR (enrofloxacin), NOR (norfloxacin), and CIP (ciprofloxacin). The drug resistance results referred to the CLSI (Clinical and Laboratory Standards Institute, USA) standards (SPE: MIC≥128 μg/mL; GEN,KAN: MIC≥32 μg/mL; ENR,NOR,CIP: MIC≥4 μg/mL) (24).

**Cross-Resistance Test**

The highly resistant strains of *M. bovis* in vitro were induced until there was 512 μg/mL of SPE, GEN, KAN, NOR, CIP, and 256 μg/mL of ENR. There were only three highly resistant strains induced successfully (Tibet-1, Tibet-6, and Tibet-8), and named *M. bovis* 1, *M. bovis* 6, and *M. bovis* 8). The highly resistant strains to ENR, NOR, and CIP were tested for cross-resistance for the highly resistant stains to SPE, GEN, and KAN.

**Antibiotic Target Mutation Analysis**

The DNA was extracted by water-boiling and amplified by PCR for the sensitive, resistant, and *in vitro*-induced strains. The PCR products were recovered using the Gel Extraction Kit (Omega Bio-Tek Co., Ltd., USA) and connected to the pMD18-T. The recombinant plasmid was verified by M13 and sequenced by Shenggong Bioengineering (Shanghai) Co., Ltd. DNAMAN software was used to compare and analyze the sequencing results.

**Overexpression of the Active Efflux System of *M. bovis***

The MIC of CCCP (carbonyl cyanide m-chlorophenyl hydrazone) and VP (verapamil) was detected for the sensitive, resistant, and *in vitro*-induced strains. The overexpression of the active efflux system was judged to exist when the MIC of antibiotics using CCCP and VP was less than 1/4 of the original MIC value.

| TABLE 1 | Primer sequences. |
| --- | --- |
| **Gene name** | **Primer sequence (5′ → 3′)** | **Tm (°C)** | **Product length** |
| rs 3 | F: GGATATCTGACGCGGTGTC<br>R: CGTCTTGATGAAGGATACCT | 50°C | 1,857 bp |
| rs 4 | F: GAAGTTTGACGGCCTGCTC<br>R: GTATTTTCTTAGTGTTCGA | 43°C | 1,812 bp |
| rps E | F: GCATGGAGCTTGTGCAAAAGA<br>R: CCGTGCCTTAAACCAAAAGGTC | 51°C | 968 bp |
| gyr A | F: GACGAATCTCATCCAGCA<br>R: GCCCTCTGCTCCCAAAGTAGC | 56°C | 531 bp |
| gyr B | F: CCGTGTGCGATTTGTC<br>R: CCATCGACATCAACATCGTC | 56°C | 555 bp |
| par C | F: GGTACCTGCTGAGCTAAAGTG<br>R: GATTAGTGCGCCCATCGC | 56°C | 488 bp |
| par E | F: GAGCAACAGTTAAACAGATTG<br>R: GGCATAAACAACCTGCTT | 56°C | 502 bp |

**PCR reaction conditions: 95°C pre-denaturation 3 min, (95°C denaturation 30 s, Tm°C annealing 30 s, 72°C extension 45 s, 30 cycles), extension 10 min at 72°C.**

| TABLE 2 | The MIC results of the 10 strains (μg/mL). |
| --- | --- |
| **Strain name** | **MIC (μg/mL)** |
| SPE | GEN | KAN | CIP | ENR | NOR |
| Tibet-1 | 64 | 16 | 8 | 8 | 2 | 1 |
| Tibet-2 | 32 | 8 | 8 | 2 | 2 | 0.25 |
| Tibet-3 | 32 | 8 | 4 | 2 | 1 | 0.25 |
| Tibet-4 | 16 | 2 | 2 | 1 | 0.5 | 2 |
| Tibet-5 | 16 | 2 | 16 | 0.5 | 2 | 0.5 |
| Tibet-6 | 128 | 64 | 2 | 4 | 8 | 2 |
| Tibet-7 | 8 | 8 | 2 | 0.5 | 0.25 | 4 |
| Tibet-8 | 128 | 1 | 4 | 4 | 0.5 | 1 |
| Tibet-9 | 4 | 1 | 1 | 1 | 0.25 | 4 |
| Tibet-10 | 4 | 2 | 8 | 0.5 | 1 | 0.5 |

SPE, spectinomycin; GEN, gentamicin; KAN, kanamycin; CIP, ciprofloxacin; ENR, enrofloxacin; NOR, norfloxacin.
TABLE 3 | Test results of cross-resistance induced in vitro (µg/mL).

| Strain name | Inducing drug | MIC (µg/mL) | Inducing drug | MIC (µg/mL) |
|-------------|---------------|-------------|---------------|-------------|
|              | concentration µg/mL | CIP | ENR | NOR | concentration µg/mL | SPE | GEN | KAN |
| M. bovis 1   | CIP (512)       | 512 | 128 | 128 | SPE (512)       | 512 | 128 | 128 |
|              | ENR (256)       | 128 | 256 | 64  | GEN (512)       | 128 | 512 | 64  |
|              | NOR (512)       | 64  | 64  | 512 | KAN (512)       | 128 | 64  | 512 |
| M. bovis 6   | CIP (512)       | 512 | 64  | 256 | SPE (512)       | 512 | 256 | 64  |
|              | ENR (256)       | 256 | 256 | 256 | GEN (512)       | 128 | 512 | 32  |
|              | NOR (512)       | 128 | 32  | 512 | KAN (512)       | 256 | 256 | 512 |
| M. bovis 8   | CIP (512)       | 512 | 256 | 256 | SPE (512)       | 512 | 256 | 256 |
|              | ENR (256)       | 32  | 256 | 128 | GEN (512)       | 128 | 512 | 64  |
|              | NOR (512)       | 128 | 64  | 512 | KAN (512)       | 128 | 128 | 512 |

SPE, spectinomycin; GEN, gentamicin; KAN, kanamycin; CIP, ciprofloxacin; ENR, enrofloxacin; NOR, norfloxacin. M. bovis 1, M. bovis 6, M. bovis 8: the highly resistant strains induced in vitro.

RESULTS

Antibiotic Susceptibility Testing
The MIC results of the 10 strains showed that Tibet-1 was resistant to CIP; Tibet-6 was resistant to SPE, GEN, CIP, and ENR; Tibet-7 was resistant to NOR; Tibet-8 was resistant to SPE and CIP; Tibet-9 was resistant to NOR; and other isolates were relatively sensitive to these antibiotics (Table 2).

Detection of Cross-Resistance
Three resistant strains to one fluoroquinolone antibiotic were used for cross-resistance to the other fluoroquinolone antibiotics and the same for aminoglycosides. The results showed that there was cross-resistance (Table 3).

PCR Results of Target Genes
There were 531 bp, 555 bp, 488 bp, and 502 bp fragments in gyrA, gyrB, parC, and parE genes for the susceptible strains, drug-resistant strains, and in vitro-induced strains to fluoroquinolones (Figure 1); there were 1,857, 1,812, and 696 bp fragments in rrs3, rrs4, and rpsE genes for the susceptible strains, drug-resistant strains, and in vitro-induced strains to aminoglycosides (Figure 2).

Analysis of Target Gene Mutations
The mutation analysis of gyrA, gyrB, parC, and parE showed that there was a nonsense mutation in parC (GAC84GAT) of clinically sensitive strains. Mutated amino acids Ser83Phe or Tyr, Ser80Ile or Arg, or Ser81Phe were detected due to base mutations...
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FIGURE 2 | PCR results of drug-resistant target genes rrs3 (A), rrs4 (B), and rpsE (C). M: DL2000; N: Negative control; 1~10: Tibet 1-10; 11~13: SPE-inducible strains; 14~16: GEN-inducible strains; 17~19: KAN-inducible strains.

TABLE 4 | The QRDR mutations of clinical drug-resistant strains.

| Strain name | Gene mutation site situation |
|-------------|-----------------------------|
|              | gyrA Ser83(TCT)             |
| Tibet-1 CIP | Phe (TTT)                   |
| Tibet-6 CIP | -                           |
| Tibet-8 CIP | Phe (TTT)                   |
| Tibet-6 ENR | Tyr (TAT)                   |
| Tibet-7 NOR | -                           |
| Tibet-9 NOR | -                           |
|              | gyrB Ser 80 (AGT)           |
| Tibet-1 CIP | -                           |
| Tibet-6 CIP | Ile (ATT)                   |
| Tibet-8 CIP | -                           |
| Tibet-7 ENR | -                           |
| Tibet-9 NOR | -                           |
|              | parC Ser 81 (TCT)           |
| Tibet-1 CIP | -                           |
| Tibet-6 CIP | -                           |
| Tibet-8 CIP | -                           |
| Tibet-7 ENR | Phe (TTT)                   |
| Tibet-9 NOR | -                           |

Tibet-1 CIP: The clinical drug-resistant strain to ciprofloxacin; the same for Tibet-6 CIP; Tibet-8 CIP; Tibet-6 ENR; Tibet-7 NOR; Tibet-9 NOR.

The Result of the Active Efflux System
The MIC of six antibiotics had no changes after using CCCP and VP, the result showed that there was no active efflux system based on fluoroquinolones and aminoglycosides expressed in M. bovis from Tibet yaks (Tables 7, 8).

DISCUSSION

M. bovis is one of the most important pathogens that causes bovine respiratory syndrome (25). Therefore, M. bovis depends on drug treatment without a commercial vaccine outside the United States (4). The strain is relatively sensitive to aminoglycosides and fluoroquinolones. However, the large-scale use of antibiotics is contributing to the development of resistance. The action of the drug can be blunted due to drug resistance, thus affecting the health of animals and human (12).

In our study, the antibiotic susceptibility testing showed Tibet-1 was resistant to CIP; Tibet-6 was resistant to SPE, GEN, CIP, and ENR; Tibet-7 was resistant to NOR; Tibet-8 was resistant to SPE and CIP; and Tibet-9 was resistant to NOR. Half of the isolated strains were detected to be drug-resistant and Tibet-6 was resistant to four antibiotics. The drug resistance of M. bovis from yaks could not be ignored.

There was limited information about the resistance mechanism of M. bovis from yaks. In our research, the
studies and further confirmed the drug-resistance mechanism of aminoglycosides. The result was the same as that of previous one of M. bovis rRNA due to inhibition of polypeptide synthesis (with the binding between aminoglycosides and the site in 16S encoding ribosomal protein S5. The strains were disrupted in rrs3, and a sense mutation was not detected in the rpsE-in rrs3 and rrs4, two strains had a C1192T base mutation in rrs3 and rrs4 of the highly resistant strain to KAN; and one resistant strain to GEN; there was a base mutation of A1408G in rrs3 and rrs4 of the highly resistant strain to SPE C1192T - - M. bovis 1 CIP, The high drug-resistant strains induced in vitro to ciprofloxacin; the same for the others.

TABLE 5 | The QRDR mutations of strains induced in vitro to fluoroquinolones.

| Strain name | Gene mutation site situation |
|-------------|-----------------------------|
| M. bovis 1 CIP | Gly 81 (GGT) Ser 83 (TCT) Glu 87 (GAA) |
| M. bovis 1 ENR | Phe (TTT) |
| M. bovis 1 NOR | Phe (TTT) |
| M. bovis 6 CIP | Cys (TGT) Asp (GAT) |
| M. bovis 6 ENR | Phe (TTT) |
| M. bovis 6 NOR | Phe (TTT) |
| M. bovis 8 CIP | Lys (AAA) |
| M. bovis 8 ENR | Lys (AAA) |
| M. bovis 8 NOR | Phe (TTT) |

M. bovis 1 GEN, The high drug-resistant strains induced in vitro to gentamicin; the same for the others.

TABLE 6 | The mutations of target genes in strains induced in vitro to aminoglycosides.

| Strain name | Gene mutation site situation |
|-------------|-----------------------------|
| M. bovis 1 GEN | rrs 3 |
| M. bovis 1 KAN | A1409G |
| M. bovis 1 SPE | A1408T |
| M. bovis 6 GEN | A1409G |
| M. bovis 6 KAN | A1408T |
| M. bovis 6 SPE | A1409G |
| M. bovis 8 GEN | A1408T |
| M. bovis 8 KAN | A1408T |
| M. bovis 8 SPE | C1192T |

M. bovis 1 GEN, The high drug-resistant strains induced in vitro to gentamicin; the same for the others.

single mutation analysis of rrs 3, rrs 4, and rps E showed that there was no base mutation in clinically sensitive and resistant strains to aminoglycosides. There were base mutations in rrs 3 and rrs 4 of nine strains induced in vitro: there was a base mutation of A1409T in rrs 3 and rrs 4 of the highly resistant strain to GEN; there was a base mutation of A1408G in rrs3 and rrs4 of the highly resistant strain to KAN; and one highly resistant strain to SPE had a C1192T base mutation in rrs3 and rrs4, two strains had a C1192T base mutation in rrs3, and a sense mutation was not detected in the rpsE-encoding ribosomal protein S5. The strains were disrupted with the binding between aminoglycosides and the site in 16S rRNA due to inhibition of polypeptide synthesis. Base mutation and the corresponding amino acid mutation have been associated with aminoglycoside resistance. Thus, we concluded that the single base mutation of 16Sr RNA (rrs3 or rrs4) would mediate the resistance of M. bovis from yaks to aminoglycosides. The result was the same as that of previous studies and further confirmed the drug-resistance mechanism of M. bovis to aminoglycosides. The isolated strains of M. bovis from yaks in Tibet had varying degrees of resistance to fluoroquinolones and aminoglycosides.
TABLE 7 | The MIC effects of fluoroquinolones by using CCCP and VP.

| Strain  | CIP MIC (ug/mL) | CCCP MIC (ug/mL) | VP MIC (ug/mL) | ENR MIC (ug/mL) |
|---------|-----------------|------------------|---------------|-----------------|
| Tibet-1 | 8               | 8                | 8             | 2               |
| Tibet-2 | 2               | 2                | 2             | 2               |
| Tibet-3 | 2               | 2                | 2             | 1               |
| Tibet-4 | 1               | 1                | 1             | 0.5             |
| Tibet-5 | 0.5             | 0.5              | 0.5           | 2               |
| Tibet-6 | 4               | 4                | 4             | 8               |
| Tibet-7 | 0.5             | 0.5              | 0.5           | 0.25            |
| Tibet-8 | 4               | 4                | 4             | 0.5             |
| Tibet-9 | 1               | 1                | 1             | 0.25            |
| Tibet-10| 0.5             | 0.5              | 0.5           | 1               |
| M. bovis 1 | 512          | 512              | 512           | 256             |
| M. bovis 6 | 512           | 512              | 512           | 256             |
| M. bovis 8 | 512           | 512              | 512           | 256             |

Tibet 1-10: the clinical strains; M. bovis 1, 6, and 8: the highly resistant strains induced in vitro.

TABLE 8 | The MIC effects of aminoglycosides by using CCCP and VP.

| Strain  | SPE MIC (ug/mL) | CCCP MIC (ug/mL) | VP MIC (ug/mL) | GEN MIC (ug/mL) |
|---------|-----------------|------------------|---------------|-----------------|
| Tibet-1 | 64              | 64               | 64            | 16              |
| Tibet-2 | 32              | 32               | 32            | 8               |
| Tibet-3 | 32              | 32               | 32            | 8               |
| Tibet-4 | 16              | 16               | 16            | 2               |
| Tibet-5 | 16              | 16               | 16            | 2               |
| Tibet-6 | 128             | 128              | 128           | 64              |
| Tibet-7 | 8               | 8                | 8             | 8               |
| Tibet-8 | 128             | 128              | 128           | 1               |
| Tibet-9 | 4               | 4                | 4             | 1               |
| Tibet-10| 4               | 4                | 4             | 2               |
| M. bovis 1 | 512           | 512              | 512           | 512             |
| M. bovis 6 | 512           | 512              | 512           | 512             |
| M. bovis 8 | 512           | 512              | 512           | 512             |

Tibet 1-10: the clinical strains; M. bovis 1, 6, and 8: the highly resistant strains induced in vitro.

The mechanism of resistance to fluoroquinolones and aminoglycosides was identified preliminarily for M. bovis from yaks: the single-site base mutation mediated the resistance of M. bovis from yaks and both base mutations led to the highly resistant strain (aminoglycosides: rrs3 and rrs4; fluoroquinolones: gyrA and parC). The research could provide a reference for clinical treatment of Mycoplasma bovis.

AUTHOR CONTRIBUTIONS

JN and SS conceived and designed the study. JN and MY executed the experiment and analyzed the sera and tissue samples. ZC, JX, and YX analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual content, and approved the final version.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

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