NOTE *Bacteriology*

Antimicrobial susceptibility and molecular characterization of macrolide resistance of *Mycoplasma bovis* isolates from multiple provinces in China

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ABSTRACT. *Mycoplasma bovis* has spread widely throughout the world via animal movement and has become an important pathogen of bovine respiratory disease. However, the minimum inhibitory concentrations of antimicrobials for *Mycoplasma bovis* have not been studied in China. The objective of this study was to determine the prevalence and antibiotic resistance of *Mycoplasma bovis* isolated from young cattle with respiratory infection in China. *Mycoplasma bovis* was detected in 32/45 bovine respiratory infection outbreaks at beef farms in 8 provinces in China. The isolates were susceptible or had medium sensitivity to ciprofloxacin, enrofloxacin and doxycycline, but were frequently resistant to macrolides (13/32, 41%). An A2058G (*Escherichia coli* Numbering) mutation located in the *rrnA* operon in domain V of 23S rRNA was observed in strains that were resistant to macrolides. This single mutations at the rrnA operon in domain V of 23S rRNA may play an important role in the resistance of *Mycoplasma bovis* strains to macrolides.

KEY WORDS: antimicrobial susceptibility, bovine respiratory infection, macrolides, *mycoplasma bovis*, target mutation

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Mycoplasma bovis (*M. bovis*) was first isolated from a severe case of mastitis in cattle in the United States in 1961 [7]. Subsequently, the results of some studies have suggested that *M. bovis* can cause respiratory infection, arthritis and tenosynovitis in feedlot cattle [2]. In recent years, *M. bovis* has spread widely to all parts of the world via animal movement and has become an important pathogen of bovine respiratory disease (BRD) in China and other countries [5]. The BRD caused by *M. bovis* is mainly treated with antibiotics, including veterinary macrolide antibiotics and fluoroquinolones in China, but treatment of BRD with macrolides often fails, leading to important economical losses in China.

Macrolide resistance has been described for pathogens of BRD, including *M. bovis*, *Pasteurella multocida* and *Mannheimia haemolytica*, in different countries. However, we found that the macrolide resistance of these pathogens is quite different in different countries. The genes *msr*(E) *mph*(E) and *erm*(42) have been shown to confer resistance to macrolides in *Pasteurella multocida* and *Mannheimia haemolytica* in Germany [11]. However, high-level macrolide resistance of *Pasteurella multocida* and *Mannheimia haemolytica* isolated in Europe can be due to 23S rRNA mutations [12]. One study of *M. bovis* isolated in Israel found that a combination of mutations in two domains of 23S rRNA is necessary to achieve higher minimum inhibitory concentrations of macrolides (MICs, ≥128 μg/m*l*) [8]. However, little research on this topic has been conducted in China. Therefore, systematic monitoring of antibiotic susceptibility and determination of the macrolide resistance mechanism of *M. bovis* strains in China are important.

A total of 32 *M. bovis* strains originating from 32 feedlot cattle herds located in 8 provinces in China (Jilin, Heilongjiang, Neimenggu, Liaoning, Shandong, Hebei, Henan and Jiangsu) were tested in this study. Samples from lungs and nasal swabs were collected from distinct outbreaks between 2011 and 2013. All samples were incubated on pleuropneumonia-like organism (PPLO) agar plates. Suspected colonies with typical "fried egg" morphology were selected from each sample and identified using biochemical methods and PCR assay, as described previously [13]. The number of color changing units (CCU) was calculated, and antimicrobial susceptibility testing was performed by the microplate dilution method [4, 10]. Two-fold dilutions of antibiotics from 0.03 to 256 μg/m*l* were tested. Because there are no CLSI-approved MIC cut-off values for veterinary *Mycoplasma species*, it is difficult to interpret the impact of antimicrobial activity *in vitro*. CLSI-approved interpretative criteria for other respiratory bovine pathogens are frequently used to understand the implication of *M. bovis* sensitivity testing *in vitro* [6, 14]. The cut-off values for enrofloxacin and ciprofloxacin (susceptible, ≤0.25 μg/m*l*; resistant, ≥2 μg/m*l*), doxycycline (susceptible, ≤4 μg/m*l*; resistant, ≥16 μg/m*l*), clindamycin (susceptible, ≤0.5 μg/m*l*; resistant, ≥4 μg/m*l*), tulathromycin (susceptible, ≤16 μg/m*l*; resistant, ≥64 μg/m*l*) and florfenicol (susceptible, ≤2 μg/m*l*; resistant, ≥8 μg/m*l*) were defined, and the cut-off values for macrolides (susceptible, ≤16 μg/m*l*; resistant, ≥64 μg/m*l*) were defined by dichotomizing the

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Table 1. Primers and PCR amplification protocols used in this study

Amplitication target	Primer	Primers sequence (5′–3′)	Product (bp)	Annealing Temperature (°C)
rrnA	*rrnA*-F	GGATATCTAACGCCGTGTCT	5,041	50
	rrnA-R	GTACTGGTCAGCTCAACAC		
rrnB	*rrnB*-F	GCATGCAAGGTTAAGCAG	2,848	50
	rrnB-R	CTAATTCCAAGTGCCACTAGCG		
L4	L4-F	TTTAGAAAAAAGAAATGAAGACAA	603	49
	L4-R	CTACTCATATTGGCGATCTAGTT		
L22	L22-F	ATGAGTACTCAACAAGCTAAAGCA	329	49
	L22-R	AATGCTATTGATAAATTAGATGTTC		

latest CLSI criteria for veterinary pathogenic bacteria BRD [3]. The reference strains, *Escherichia coli* ATCC 25922 and *M. bovis* type strain PG45 (ATCC 25523), were used for a quality control. To identify rRNA mutations of *M. bovis* that confer resistance to macrolides, the two alleles that contain domain II and domain V were detected. PCR was performed according to a previous study [8]. The primers and program are shown in Table 1. PCR was performed in a 25 μl reaction volume containing 2.5 μl 10 × PCR buffer, 0.5 mmol L^{-1} dNTP, 0.5 μmol L^{-1} of each primer, 15.6 μl PCR water and 1 U LA-Taq polymerase (Takara, Otsu, Japan). The sequences were compared with the sequence of PG45, and sequences editing, consensus and alignment construction were performed by DNASTAR and ClustalW. Numbering of nucleotide and amino acid positions is based on the 23 rRNA gene or L4/L22 proteins of *Escherichia coli*, respectively.

Then, the MIC of erythromycin was determined in the presence of the potent efflux inhibitors, carbonyl cyanide m-chlorophenyl hydrazones (CCCPs) and verapamil, at appropriate concentrations using the broth microdilution method [17]. Some previous studies have found that MICs change in the presence or absence of inhibitors due to changes in the level of activity of efflux pumps. However, a two-fold reduction is not sufficient to rule out false positives. Thus, a four-fold or greater reduction in strain MICs in the presence of inhibitors is considered to be due to the activity of efflux pumps [9, 18]. Each experiment was repeated three times.

The MIC values of the 10 antimicrobial agents obtained from the examinations of the China *M. bovis* isolates are shown in Table 2, and the MIC values for PG45 were as follows: erythromycin (2 μg/m*l*), azithromycin (2 μg/m*l*), kitasamycin (2 μg/m*l*), tylosin (0.125 μg/m*l*), clindamycin (0.125 μg/m*l*), lincomycin (0.25 μg/m*l*), doxycycline (0.03 μg/m*l*), ciprofloxacin (0.125 μg/m*l*), enrofloxacin (0.125 μg/m*l*) and florfenicol (2 μg/m*l*). Fluoroquinolones were found to be the most active compounds *in vitro* (MIC ≤1 μg/m*l*). For one isolate, the MIC for florfenicol was high (MIC=8 μg/m*l*), while the rest of the strains were inhibited by florfenicol at lower concentrations (MIC ≤4 μg/m*l*). Thirteen (41%) isolates were resistant to erythromycin, tylosin, azithromycin and kitasamycin, with MICs ≥64 μg/m*l*. The MICs of clindamycin and lincomycin were different in two distinct populations of isolates: 13 strains yielded MICs ≥128 μg/m*l*, while the rest yielded MICs ≤0.125 μg/m*l*. These results are in accordance with previous studies in other countries, which found that the most active compounds were fluoroquinolones and florfenicol [15, 16]. However, macrolides have been used traditionally and are losing their efficacy against *M. bovis* in many countries, which is in accordance with our results, as many *M. bovis* strains in China are already resistant to macrolides. Furthermore, the strains that were resistant to macrolides were also resistant to lincomycin and clindamycin, which has been previously observed in other *Mycoplasma* of animal and human origins. The mechanism of this resistance probably involves rRNA mutations [1, 10].

The 23S rRNA gene sequences of susceptible strains and resistance strains were analyzed (Table 3). The macrolide-resistant strains only had one mutation type, an A2058G substitution in domain V in the *rrnA* operon of the 23S rRNA. None of the macrolide-resistant strains contained substitutions in the *rrnB* operon. Additionally, there were no significant differences in domain II of L4 or L22 ribosomal proteins between resistant and susceptibl isolates. The A2058G substitution in domain V was the most prevalent substitution in our study and a previous study [8], and this single mutation may play an important role in the resistance of *M. bovis* strains to macrolides. However, other point mutations were found in previous studies in one or two domains of 23SrRNA, including G748A, C752T, A2059G and A2059C, which can reduce the sensitivity of *M. bovis* to tylosin and tilmicosin. Differences in the genotypes of different *M. bovis* strains may be due to differences in their evolutionary courses in different countries or to the development of different resistance mechanisms. Our results indicate that the values of erythromycin and azithromycin were not decreased in the presence of CCCP (32 μg/m*l* and 64 μg/m*l* for both antibiotics, respectively) or verapamil (64 μg/m*l* and 128 μg/m*l*, respectively) in highly resistant strains, and Chinese macrolide-resistant strains were negative for antibiotic efflux. Whether *M. bovis* strains have other resistance mechanisms should be investigated further in future studies.

This is the first report of systematic monitoring of antibiotic susceptibility of *M. bovis* in China. We believe that studies should be performed to evaluate changes in MIC values and genetic mutations to determine the prevalence of *M. bovis* strains that are resistant to different antimicrobials.

Table 2. MICs distribution of *M. bovis* isolates

Antimicrobial agents[a)]	No. of isolates with MIC (μg/ml)[b)]													
	≤0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	≥256
ERY										10	9			13
AZM				3	5	3	6	2						13
KIT						1	9	8	1			10	3	
Tm						4		6	5	4		8	5	
TYL									5	6	8	5	6	2
CLI	3	6	5		5								10	3
LIN		2	4	4	3	6						4	7	2
DOX	1			4	15	12								
CIP			2	10	16	4								
ENR	1		17	9	5									
FFC							19	12	1					

a) ERY=erythromycin; AZM=azithromycin; KIT=kitasamycin; Tm=tilmicosin; TYL=tylosin; CLI=clindamycin; LIN=lincomycin; DOX=doxycycline; CIP=ciprofloxacin; ENR=enrofloxacin; FFC=florfenicol. b) Cut-off values were used according to CLSI document VET01-A4 for other respiratory bovine pathogens. Full vertical lines indicate the cut-off between intermediate and resistant strains. Dotted vertical lines indicate the cut-off between susceptible and intermediate strains.

Table 3. Molecular characterization of macrolides-resistant *M. bovis* field isolates

Isolate types	No. of isolates	23S rRNA Domain II *rrnA*	23S rRNA Domain V *rrnA*	23S rRNA Domain II *rrnB*	23S rRNA Domain V *rrnB*	L4 ribosomal protein	L22 ribosomal protein
resistant isolates	13	None	A2058G	None	None	None	None
susceptible isolates	19	None	None	None	None	None	None

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