

# Methylase Genes-Mediated Erythromycin Resistance in *Staphylococcus aureus* from Bovine Mastitis in China

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## ABSTRACT

Erythromycin is commonly used to treat bovine mastitis caused by *Staphylococcus aureus* (*S. aureus*), which has resulted in a high-level of resistance in China. In this study, we investigated erythromycin-resistant phenotypes and relevant resistant genes among 26 *S. aureus* isolates from bovine mastitis in China. Twenty (76.9%) of erythromycin-resistant isolates were identified (MIC values  $\geq 64$   $\mu\text{g}/\text{mL}$ ). Resistance phenotype analysis determined 15 (57.7%) inducible-macrolide, lincosamide, and streptogramin B (iMLS<sub>B</sub>) and 5 (19.2%) constitutive macrolide, lincosamide, and streptogramin B (cMLS<sub>B</sub>) phenotypes, respectively. PCR detection of efflux genes (*mefA* and *msrA*) and methylase genes (*ermA*, *ermB* and *ermC*) confirmed that none of *mefA*, *msrA*, and *ermA* genes, which were demonstrated exhibiting the efflux mechanism existed in the resistant isolates. However, *ermB* genes were determined in 100% (26/26) and *ermC* genes were detected in 84.6% (22/26). These findings suggested that erythromycin-resistance was caused mainly by methylase encoded by *ermB* and *ermC* genes. Interestingly, 6 erythromycin-susceptible isolates were presented in *ermB*-carrying isolates, and 2 were detected in both *ermB*- and *ermC*-carrying isolates. Further sequence analysis of the *ermB*, *ermC* and 23S-rRNA genes revealed that erythromycin-susceptibility might be engendered by mutations of 23S-rRNA resulting in an inability for methylation. These data adequately illustrated that ribosome methylases encoded by *ermB* and *ermC* genes has played a vital role in the resistance of *S. aureus* in bovine mastitis in China.

**Keywords:** *Staphylococcus aureus*, efflux mechanism, erythromycin resistance, methylase genes.

## INTRODUCTION

Mastitis, an important disease of dairy cows, occurs widely and can be caused by infections with bacteria, yeast or fungi (1,2). A recent investigation showed that *Staphylococcus aureus* (*S. aureus*) represented a greater proportion of the isolated pathogenic bacteria in cases of clinical mastitis (3). Erythromycin, a macrolide antibiotic, is effective against a variety of gram positive bacteria and commonly used to treat

bovine mastitis (3,4). As a result of widespread utilization of erythromycin, the resistance level of *S. aureus* has been increasing rapidly. Therefore an investigation into the erythromycin-resistance of *S. aureus* has become imperative (5).

Previous studies have reported that the majority of resistance to erythromycin from different cases of bovine mastitis may be due to different mechanisms: drug inactivation (6), loss of permeability (7), an active efflux mechanism (8) or tar-

get region modification by methylation or mutation (9). One of the important active efflux mechanisms may be based on the membrane-bound efflux protein encoded by *mefA* gene (10). Another is involved in macrolides-streptogramins resistance gene A (*msrA*) which emerged in clinical strains of *S. aureus* since the 1980's (11). *msrA* gene encodes macrolide efflux pump which is frequently detected in *S. aureus*, belongs to the ABC transporter family, and induces resistance to 14- and 15-membered macrolides (M phenotype) and streptogramins B only (12).

The structural changes in ribosomal RNA (rRNA) that prevent the binding of macrolides are another important resistance mechanism conferring high-level resistance. (13). *S. aureus* is able to synthesize such kind of enzyme: ribosome methylases, which are encoded by one or more erythromycin-resistant methylase genes (*erm*). After the methylation of 23S-rRNA, the binding site for macrolide-lincosamide-streptogramin B (MLS<sub>B</sub>) antibiotics is altered. *erm* genes often confer constitutive resistance to macrolides-lincosamides-streptogramin B phenotype (cMLS<sub>B</sub>), but also may encode inducible resistance phenotype (iMLS<sub>B</sub>) (14). The main methylase genes that have been identified in *S. aureus* are *ermA*, *ermB* and *ermC* (9). Production of methylase results in the N<sup>6</sup>-dimethylation of adenine residue at position 2058 of 23S-rRNA. The conformational changes which occur in the P site of 23S-rRNA prevent macrolide binding, thus the inhibitory effect of the macrolide on protein synthesis is overcome (15).

Wang *et al.* (16) reported that the prevalence of *S. aureus* exceed more than 25.2% of cases. However, there have been few studies to identify the macrolide-resistant genes in *S. aureus* isolated from bovine mastitis in China. The data regarding the investigation of the cMLS<sub>B</sub> phenotype and iMLS<sub>B</sub> phenotype has been very limited (2). Methylase genes-mediated resistance to erythromycin in *S. aureus* requires elucidating. Moreover, whether the mutation of 23S-rRNA in *S. aureus* would alter the susceptibility to erythromycin remains elusive (17).

In this study, 20 cases of erythromycin resistance in 26 clinical *S. aureus* isolates were determined. We further detected the presence of two important efflux genes (*mefA* and *msrA*) and three macrolide-resistant genes (*ermA*, *ermB* and *ermC*), as well as the influence of mutation in 23S-rRNA on the resistant isolates. This comprehensive understanding about the erythromycin-resistant mechanism could provide

clues for developing new strategies to treat clinical bovine mastitis caused by *S. aureus* in China.

## MATERIALS AND METHODS

### Bacterial strains and standard strains

During 2008-2010, 150 milk samples from 12 dairy farms were collected from clinical bovine mastitis cases in the provinces of Hebei, Hubei, and Heilongjiang, Tianjin Municipality, and Inner Mongolia autonomous region of China based on one isolates per herd. A total of 26 isolates were identified as *S. aureus* by conventional microbiological methods including gram stain, colony morphology, and coagulase testing with rabbit plasma, as well as species-specific and the ubiquitous DNA-based assay reported by Martineau *et al.* (18). The confirmed *S. aureus* isolates were stored at -20°C. The standard *Staphylococcal* strains (*ATCC25923* and *ATCC29213*) were purchased from Tianhe Biological Products Co. Ltd, Hangzhou, China.

### Testing erythromycin susceptibility of *S. aureus*

According to the guidelines of the clinical and laboratory standards institute (19), Kirby-Bauer antibiotic testing method (K-B testing) was performed for evaluating susceptibility of *S. aureus* to erythromycin. *S. aureus* was streak inoculated onto the solidified Mueller-Hinton (M-H) agar media (Hope Bio-Technology, Qingdao, China) and cultured at 37°C for 18-24 h. Then single colonies were inoculated into M-H broth cultural media for 4 h at 35°C. *S. aureus* suspension (optical density at 600 nm wavelength (OD<sub>600</sub>) arriving at 0.1) was diluted serially tenfold. The bacterial suspension (0.5 mL) was seeded onto a 90-mm-diameter solidified M-H agar media, and antimicrobial susceptibility test discs (Tianhe microorganism, Hangzhou, China) were placed onto the agar media. These dishes were incubated for 16-18 h at 35°C, followed by determination of susceptibility through measuring the diameter of inhibitory zone for bacterial growth around the test discs. The *S. aureus* was interpreted as sensitive (S), intermediate (I), and resistant (R) when the inhibitory diameter were ≥ 23 mm, 14-23 mm, and ≤ 14 mm, respectively (20). Each strain was repeated three times and a control test was performed with the standard strain (*ATCC25923*). At the same time, in light of the above method, the broth dilution method (serial double dilution) (19) was performed in order to determine the minimal inhibitory concentration (MIC) of

erythromycin for the resistant isolates and standard strain (*ATCC29213*) which was used as the control strain.

### Determining the bacterial active efflux mechanism

In the process to determine the MIC values, the broth cultural media containing 20 µg/mL efflux inhibitor Reserpine (Tianhe, Hangzhou, China) was prepared to estimate the effects of efflux inhibitor on the erythromycin resistance. The tested bacterium was interpreted as exhibiting the active efflux mechanism when the MIC value in broth cultural media containing reserpine and erythromycin was four times lower than the MIC value containing only erythromycin (21).

### Analyzing the resistant phenotypes

A double-disc agar diffusion test (D-test) (22) was performed to determine the resistance phenotype of erythromycin-resistant isolates. After fresh growing bacteria were inoculated onto M-H agar plates, erythromycin (15 µg) and clindamycin (2 µg), (Tianhe, Hangzhou, China) discs were placed 15 mm apart on the plates and incubated for 18 to 24 h at 35°C. Resistance to erythromycin and a “D”-shaped zone around the clindamycin disc was defined as iMLS<sub>B</sub> phenotype. Resistance to both antibiotics was defined as the cMLS<sub>B</sub> phenotype (4).

### PCR detecting *mefA*, *msrA*, *erm*, and 23S-rDNA genes

To begin with, the proteinase (K) method (8) was applied for extracting bacterial genomic DNA. In brief, 1.5 mL *S. aureus* suspension was centrifuged (CR21 Hitachi, Tokyo, Japan) at 12,000 rpm for 1 min. The bacterial pellets were collected, then 600 µL Tris-EDTA (TE) buffer, 20 µL 100 mg/mL lysozyme (Invitrogen, CA, USA), 20 µL 10 mg/mL RNase (Invitrogen, CA, USA), were added and incubated at 37°C for 2.5 h, followed by an addition of 70 µL 10% SDS and 5 µL 20 mg/mL proteinase K (Sigma, New York, USA) at 37°C for 1 h. Subsequently, supernatant was extracted with phenol/chloroform/isoamyl alcohol (25: 24: 1) and centrifuged at 12,000 rpm for 5 min. Finally, via isopropanol precipitation, washing with 70% ethanol, centrifugation and drying in sequence, the obtained DNA was diluted in 50 µL

**Table 1:** Primers used to detect the erythromycin-resistant genes.

Genes	Primer	PCR product size
<i>msrA</i>	F: 5'-CGATGAAGGAGGATTTAAAATG-3' R: 5'-CATGAATAGATTGTCCTGTTAATT-3'	1733 bp
<i>mefA</i>	F: 5'- AGTATCATTAACTACTAGTGC -3' R:5'-TTCTTCTGGTACAAAAGTGG-3'	367 bp
<i>ermA</i>	F: 5'-ATGAACCAGAAAAACCCTAAA-3' R:5'-GGCTTAGTGAAACAATTTGTAAC-3'	732 bp
<i>ermB</i>	F: 5'-GGCGGATGAACAAAAATATAAAAATA-3' R:5'-GCGTTATTTCCCTCCCGTTAAA-3'	738 bp
<i>ermC</i>	F: 5'-GGCATGAACGAGAAAAATATAAAA-3' R:5'-GCGGGTTACTTATTAATAAATTT-3'	735 bp
<i>23S-rDNA</i>	F: 5'-GGTGAGCTCGATTAAGTTATTAAGGG-3' R:5'-CTCGTCGACTAACCTCATCATCTTTG-3'	2854 bp
<i>ermB P</i>	F: 5'-GGGGATCCTGTATAATAGGAATTGA-3' R:5'-GTACTCGAGTTATTTCCCTCCCGTT-3'	1011 bp
<i>ermC P</i>	F: 5'-GCGGGATCCTGTTCATATTTATC-3' R:5'-GGGGGCTCGAGTTACTTATTAATA-3'	913 bp

buffer. In addition, GTpure™ Kit (Gene Tech, Shanghai, China) was used to extract bacterial plasmids according to the product manual. Furthermore, a modified version of the PCR gene detection method described by Huang *et al.* (23) was performed. We used one pair of primers (synthesized by Sangon Biotech, Shanghai, China) for *msrA*, *mefA*, *ermA*, *ermB*, *ermC*, *ermB* promotor (*ermB P*), *ermC* promotor (*ermC P*), and 23 *S-rDNA* genes, respectively. The primer sequences were shown in Table 1. The 2×EasyTaq PCR Supermix systems (Takara, Dalian, China) were used for amplification of *msrA*, *mefA*, *ermA*, *ermB*, and *ermC*. The PCR reaction contained 2×Easy Taq PCR Supermix 10 µL, 25 pM forward primer 1 µL, 25 pM reverse primer 1 µL, DNA Termplet 1 µL, and double distilled H<sub>2</sub>O 7 µL with 20 µL of the total volume. Different from the above reaction system, Fast *Pfu* enzyme (NEB, London, England) was applied for amplifying *ermB P*, *ermC P* and 23*S-rDNA* genes. The same reaction system was used for amplification of 23*S-rDNA* genes, whereas the following PCR system were used to amplify *ermB P* and *ermC P* genes: 5×PCR buffer 10 µL, 2.5 mM dNTPs 5 µL, 25 pM forward primer 1 µL, 25 pM reverse primer 1 µL, DNA Termplet 1 µL, 5 U/µL Fast *Pfu* polymerase 1 µL, ddH<sub>2</sub>O 31 µL, with 50 µL of total volume. The conditions of PCR reactions were listed in Table 2. All amplified PCR products were detected on 1% agarose gels electrophoresis and stained with ethidium bromide. PCR products were reclaimed and purified using Biospin GeL

**Table 2:** PCR programs for detecting relevant resistant genes.

Genes	Initial denaturation		Denaturation		Annealing		Extension		Final elongation		Cycles
	T(°C)	T(min)	T(°C)	T(sec)	T(°C)	T(sec)	T(°C)	T(sec)	T(°C)	T(min)	
<i>msrA</i>	94	5	94	45	50	50	72	60	72	10	30
<i>mefA</i>	94	5	94	45	48	40	72	40	72	5	30
<i>ermA</i>	94	5	94	45	52	50	72	50	72	5	30
<i>ermB</i>	94	5	94	45	53	50	72	50	72	5	30
<i>ermC</i>	94	5	94	45	52	50	72	50	72	5	30
<i>23S-rDNA</i>	94	2	94	15	53	15	72	60	72	5	30
<i>ermB P</i>	94	2	94	15	54	15	72	30	72	5	30
<i>ermC P</i>	94	2	94	15	53	15	72	30	72	5	30

Extraction Kit (Bioer, Hangzhou, China) according to the manufacture's instruction.

### Cloning *ermB* and *ermC* structural genes

For cloning the resistance determinants of *S. aureus*, PCR products were cloned into the pEASY-BLunt Vector (Takara, Dalian, China) (linking reaction composed of DNA 2.0  $\mu$ L, pEASY-BLunt Vector 1.0  $\mu$ L, mixed and incubated at 23°C for 15 min), and then transformed into the *E. coli* *Transt-t1* strain (TianHe microorganism, Hangzhou, China) following standard protocols (24). The recombinant plasmid was identified with restriction enzyme *Bam*HI and *Sal* I digestion (NEB, London, England) and 1% agarose gels electrophoresis. The obtained recombinant bacteria were cultured on agar plates containing 50 mg/ml erythromycin (Tianhe, Hangzhou, China), followed by determination of MIC changes. *Transt-t1* strain was treated as the negative

strain, while the erythromycin-resistant strain *ZJK-1* served as positive control strain.

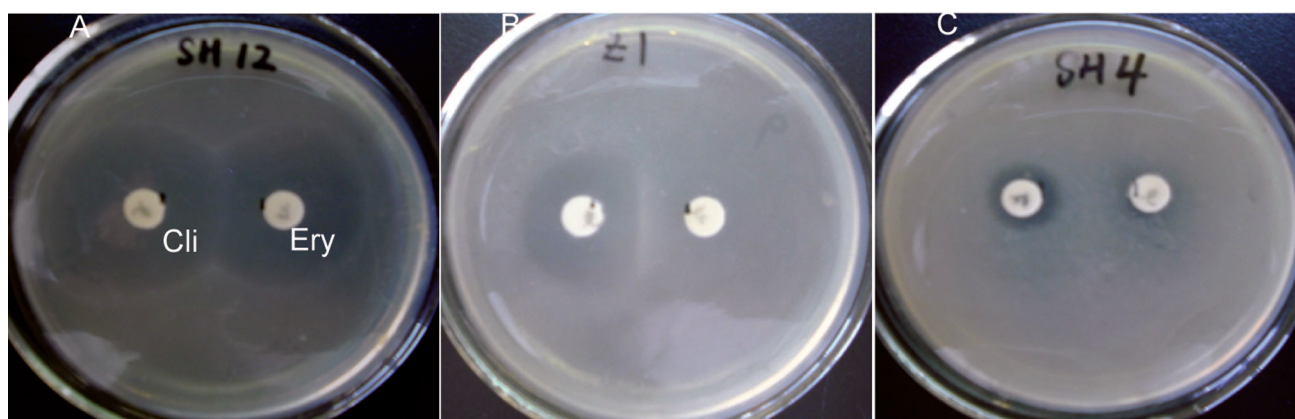
### Genes sequence analysis

Recombinant positive bacteria containing erythromycin-resistant gene were stored in 40% glycerol, and sent to Shanghai Sunny Biological Co. Ltd, China for sequencing. The sequences were edited using Sequencher™ (Gene Codes, Ann Arbor, MI) and aligned with other known sequences contained in the GenBank, and analyzed by BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) (25).

## RESULTS

### MIC distribution and active efflux mechanism

The MIC distribution of erythromycin and the bacterial phenotype are presented in Table 3. Following the criterion



**Figure 1.** The results of D-Test of *S. aureus*. Clindamycin (Cli) and erythromycin (Ery) disks were placed onto the media from left to right in each plate. A: *SH12* isolate was sensitive to both antibiotics; B: *Z1* isolate displayed resistance to erythromycin and had a clindamycin zone  $\geq$  21mm ( $D^+$ , iMLS<sub>B</sub>); C: *SH4* isolate expressed resistance to both erythromycin and clindamycin ( $D^-$ , cMLS<sub>B</sub>).

**Table 3:** Erythromycin resistance, MICs of 26 strains isolated from bovine mastitis in China.

Isolates	K-B (mm)	MIC ( $\mu\text{g}/\text{mL}$ )	Phenotype	Isolates	K-B (mm)	MIC ( $\mu\text{g}/\text{mL}$ )	Phenotype
<i>ATCC21923</i>	-	0.25	S	<i>SH-4</i>	7	128	R
<i>ATCC25923</i>	26	-	S	<i>SH-5</i>	7	128	R
<i>HLJ1</i>	6	64	R	<i>SH-6</i>	7	256	R
<i>L5-2</i>	7	256	R	<i>SH-7</i>	7	128	R
<i>HS-2</i>	6	128	R	<i>SH-8</i>	6	128	R
<i>Z1</i>	7	128	R	<i>SH-10</i>	7	128	R
<i>Z3</i>	7	128	R	<i>SH-14</i>	6	128	R
<i>Z4</i>	7	128	R	<i>SH-17</i>	6	128	R
<i>AT</i>	7	128	R	<i>HLJ23-1</i>	23	0.151	S
<i>ZJK-1</i>	6	256	R	<i>HLJ3</i>	24	0.151	S
<i>HS-4</i>	6	128	R	<i>HLJ4</i>	26	0.151	S
<i>WH-1</i>	7	256	R	<i>SH-11</i>	24	0.151	S
<i>SH-1</i>	7	128	R	<i>SH-12</i>	24	0.151	S
<i>SH-3</i>	7	128	R	<i>SH-18</i>	24	0.151	S

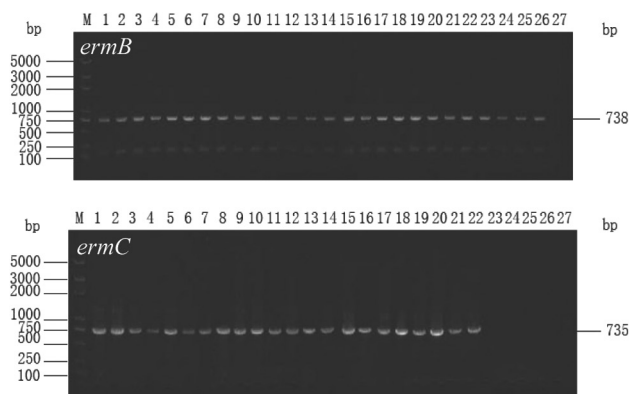
In K-B experiments, inhibitory diameter  $\geq 23\text{mm}$  was determined as sensitivity (S), 14-23mm intermediate (I),  $\leq 14\text{mm}$  resistance (R). In MIC analysis,  $< 0.5 \mu\text{g}/\text{mL}$  (S), 1-4  $\mu\text{g}/\text{mL}$  (I),  $< 8 \mu\text{g}/\text{mL}$  (R).

(20), the reference strains (*ATCC21923* and *ATCC25923*) tested in parallel with each batch of isolates, were within acceptable ranges on all occasions. There are no intermediate strains observed in susceptible experiments. Of the 26 isolates tested, only 6 (23.1%: *HLJ23-1*, *HLJ4*, *HLJ3*, *SH-11*, *SH-12*, and *SH-18*) strains were susceptible to the erythromycin. Twenty (76.9%) resistant isolates were identified by the antibiotic sensitivity test (K-B method). All the MICs of *S. aureus* strains resistant to erythromycin were also greater than  $64 \mu\text{g}/\text{mL}$ . Among the 20 stains, 19 strains were highly resistant (MIC ranged from 128 to  $256 \mu\text{g}/\text{mL}$ ), and only 1 strain had an MIC of  $64 \mu\text{g}/\text{mL}$ . Meanwhile, the 6 confirmed susceptible strains had the same MICs of  $0.151 \mu\text{g}/\text{mL}$ . Furthermore, in order to investigate whether these resistant strains were related to the active efflux mechanism, bacteria were cultured in special media with  $20 \mu\text{g}/\text{mL}$  reserpine. The MICs of erythromycin-resistant strains cultured with reserpine showed no differences ( $P > 0.05$ ) compared to the MICs of those cultured without reserpine. The results indicated that there was no effect of the efflux inhibitor on the erythromycin resistance.

### *iMLS<sub>B</sub>* and *cMLS<sub>B</sub>* resistance phenotypes

Isolates resistant to erythromycin and having a clindamycin

zone  $\geq 21\text{mm}$  with a D-shaped (blunted zone near the erythromycin disk) zone were regarded as positive for inducible resistance (D<sup>+</sup>). Isolates resistant to erythromycin and clindamycin were considered negative for the D-test (D<sup>-</sup>) (26). In this study, 6 erythromycin-susceptible strains were also susceptible to clindamycin (Figure 1A), and two phenotypes of resistance to erythromycin and cyclines were



**Figure 2.** PCR detection of the relevant resistant genes of *S. aureus* isolated from bovine mastitis in China. From left to right in sequence: Marker (2000 Plus), *HLJ1*, *L5-2*, *HS-2*, *Z1*, *Z3*, *Z4*, *AT*, *ZJK-1*, *HS-4*, *WH-1*, *SH-1*, *SH-3*, *SH-4*, *SH-5*, *SH-6*, *SH-7*, *SH-8*, *SH-10*, *H-14*, *SH-17*, *HLJ23-1*, *HLJ4*, *HLJ3*, *SH-11*, *SH-12*, *SH-18* and negative control.

observed in *S. aureus*. Among the 20 erythromycin-resistant isolates, the tested 15 isolates (75%) were susceptible to clindamycin but resistant to erythromycin, which indicated they were detected as iMLS<sub>B</sub> phenotype (Figure 1B). Whereas, the other 5 isolates (25%), resistant to the both antibiotics, were considered negative for D-test and regarded as constitutive resistance phenotypes (Figure 1C). The percentages of iMLS<sub>B</sub> phenotype and cMLS<sub>B</sub> phenotype among the 26 isolates were 57.7% and 19.2%, respectively.

### ***ermB* and *ermC* genes associated with erythromycin resistance**

The presence in all isolates of the *msrA*, *mefA*, *ermA*, *ermB*, *ermC*, *ermB P*, and *ermC P* genes responsible for erythromycin-resistance was confirmed by PCR analysis. Our analyses showed that no PCR products were amplified using *msrA*, *mefA*, and *ermA* primers for erythromycin-resistant detection. However, among the total tested isolates, 26 (*ermB*) and 22 (*ermC*) were amplified (Figure 2). The PCR detection of erythromycin-resistant genes showed that the size of amplified products detected by PCR was 738 bp (*ermB*) and 735 bp (*ermC*). The positive rates of *ermB* and *ermC* genes were (26/26) 100% and (22/26) 84.6%, respectively. The number of *ermB* and *ermC* genes coexisting in a bacterium was 22, which suggested the resistance may be related to the two genes. It was noteworthy that 6 (23.1%) of 26 *ermB*-carrying and 2 (9.1%) of 22 *ermC*-carrying *S. aureus* isolates were susceptible to erythromycin.

### **Mutation of *ermB*, *ermC* mainly occurred in structural genes**

If mutation causes a sequence alteration in a region of *ermB* and *ermC* genes or in their promotor regions, the secondary structure of methylase is also affected and may result in producing non-functional methylase or failure in its transcription and posttranscriptional modifications, which might cause susceptibility to erythromycin. In order to further investigate the reason that 6 *ermB*-carrying isolates (*HLJ23-1*, *HLJ3*, *HLJ4*, *SH-11*, *SH-12* and *SH-18*) and 2 *ermC*-carrying isolates (*HLJ23-1* and *HLJ4*) susceptible to erythromycin, *ermB*, *ermC* and their promotor genes were sequenced and their mutation analyses performed. Five erythromycin-resistant isolates carrying *erm* genes (*ZJK-1*, *HS-4*, *WH-1*, *SH-5* and *SH-8*) served as reference strains. The results of the sequence comparison are represented in Table 4. We found a

100% identity of *ermB* structural gene among *HS-4*, *HLJ4*, and transposon *Tn551* (Genebank: Y13600), and the other 9 isolates had more than 99% similarity. Among the 9 isolates, homology comparison exhibited one mutation at position 27 (A→T) in the region of *ermB P* of *SH-11* isolate, whereas the 100% identity among the other 8 isolates and the reference isolate may imply a relatively conserved status of the *ermB P* genes sequences. As for *ermC*, only one base mutation was detected in the -10 region of *ermC P* gene of *ZJK-1* isolate. Identity of 100% in the promotor region and leader peptide region among the other isolates were determined, which demonstrated that base mutation mainly located in the structural gene sequence.

### **Slight mutation of *erm* structural gene is not responsible for methylase activity**

Because the above mutation analysis indicated that a much lower base mutation occurred in the promotor region, we speculated that mutations may exist in structural genes which would be responsible for methylase activity. Accordingly, *ermB* and *ermC* genes were cloned into *pMD18-T* vector and transformed into erythromycin-susceptible bacterium (*Transt-t1*). MICs of transformed bacteria were determined. Compared with negative strains (*Transt-t1*), bacteria transformed with resistant genes displayed stronger resistant characteristics, and their MICs significantly increased to 200 µg/mL, which was interpreted that a mutation of *ermB* and *ermC* genes in the structural region did not result in inactivation of methylase.

### **Alteration of 23S-rRNA may lead to erythromycin susceptibility**

It has been reported that the induced appearance of erythromycin resistance in *S. aureus* is accompanied by methylation of the 23S-rRNA (27). However, the mutation that has been reported to affect the ribosomal structure and function has been those in which a ribosomal protein was altered. The phenotypes of these mutations have shown antibiotic resistance (28). The sequences of 23S-rRNA genes among the 6 *ermB*-carrying isolates were analyzed and the results are listed in Table 5. Homogeneity assay using DNASTAR software package (Version 7.1) indicated the 99.9% homology compared to the *Newman* strain 23S-rRNA (Genebank: AP009351). Each isolate at least contained one base mutation. Both mutations of *SH-11* and *SH-12* were located at

**Table 4:** Base changes of *ermB* and *ermC* genes.

Genes	Isolates	Promotor	Leader sequence	Structural gene
<i>ermB</i>	<i>HLJ23-1</i>		115 (A→G), 251 (A→G)	495 (C→T), 497 (T→C), 572 (A→G)
	<i>HLJ3</i>			333 (A→G), 822 (A→G)
	<i>HLJ4</i>			
	<i>SH-11</i>	27 (A→T)		662 (T→C)
	<i>SH-12</i>		251 (A→G)	435 (A→G), 497 (T→C), 572 (A→G), 871 (C→G), 913 (G→A)
	<i>SH-18</i>		251 (A→G)	495 (C→T), 497 (T→C), 572 (A→G), 871 (C→G), 913 (G→A)
<i>ermC</i>	<i>HLJ23-1</i>			485 (T→G), 643 (A→G)
	<i>HLJ4</i>			398 (T→G), 781 (A→G)

**Table 5:** Base changes of *23S-rDNA* gene.

Isolates	Base change location
<i>HLJ23-1</i>	558 (A→G), 1090 (A→G), 1243 (G→A), 1550 (G→A), 1750 (T→C) 1848 (A→G), 2312 (C→T), 2360 (A→G), 2396 (A→G), 2746 (C deletion)
<i>HLJ3</i>	182 (C→A), 1019 (A→G), 1896 (T→G), 2057 (A→G), 2569 (A→G)
<i>HLJ4</i>	44 (A→G), 2746 (G→A)
<i>SH-11</i>	989 (A→G)
<i>SH-12</i>	989 (A→G)
<i>SH-18</i>	716 (C deletion), 775 (A→G), 1192 (A→G), 1227 (T→C), 1917 (A→G), 2480 (A→T)

of the MICs of the resistant strain were  $\geq 128$   $\mu\text{g/mL}$ . The resistance rate (76.9%) was obviously higher than the results obtained from other countries (30-33), but much lower than the rate of 93.1% reported by Wang *et al.* (8). This high prevalence of erythromycin-resistant isolates may be related to high prevalence of bovine mastitis in China (2), which portends the further complication in

the treatment of *S. aureus*-induced bovine mastitis.

One significant contribution to intrinsic antibiotic resistance is provided by a number of broadly-specific multi-drug efflux systems which can play an important role in export of antibiotics (34). It is well known that reserpine is a useful efflux pump inhibitor to combat antibiotic-resistant microorganisms (21). We investigated whether this resistance was caused by the active efflux mechanism in the reserpine-resistant bacteria. The results indicated no effects of efflux inhibitor on the erythromycin resistance, which implied that the active efflux mechanism in these resistant bacteria were inactivated, or even did not exist in the resistant bacteria at all. In addition, we also distinguished susceptibility patterns of *S. aureus* between phenotypes for further testing. Constitutive resistance demonstrates resistance to all of the  $\text{MLS}_B$  groups, but inducible resistance is present with an inducing agent, such as erythromycin. Susceptibility testing of these isolates using adjacent erythromycin and clindamycin antibiotic discs demonstrated the classical D-zone to be indicative of a positive D-test in all cases (9). Previous studies have shown a variable incidence of inducible resistance among the tested Staphylococcal populations (4). In our D-test, 15 (D<sup>+</sup>, 75%)

the position 989 (A→G) of the *23S-rRNA* genes. A base deletion occurred in *SH-18* (716C) and *HLJ23-1* (C2746). We have observed that the mutation frequency of A→G were significantly higher than other base mutations. More importantly, a mutation at position 2057 (AG) of *23S-rRNA* in *HLJ3* isolate implied that the alteration of *23S-rRNA* genes may affect the methylation site.

## DISCUSSION

*S. aureus* is one of the important pathogens causing bovine mastitis. It has become a great matter of concern because more than half of the bacteria possess antibiotic resistance (5, 29). Erythromycin has been approved for the treatment of Staphylococcal mastitis in veterinary practice in China for more than half a century. Nevertheless, in the last two decades, erythromycin-resistant *S. aureus* isolates have become an increasingly recognized problem in many parts of China (8). Investigating the spread of erythromycin-resistant genes in *S. aureus* is important for controlling its dissemination in bovine mastitis. In our study, 20 of 26 *S. aureus* isolates displayed significant erythromycin resistance, and 95%

and 5 (D, 25%) isolates exhibited iMLS<sub>B</sub> and cMLS<sub>B</sub> phenotypes among the 20 resistant isolates, respectively. Rich *et al.* (35) reported 71.8% incidence of iMLS<sub>B</sub> in 285 strains from a variety of clinical infectious between January 2003 and December 2004 in UK. At the same time, the rate of our iMLS<sub>B</sub> phenotype was much higher than that (52.8%) of Inner Mongolia isolates found by Wang *et al.* (8), which were suggested that there might exist a potential risk of the failure of erythromycin and clindamycin therapy for treating *S. aureus*-induced bovine clinical mastitis.

Two phenotypes are related with resistant genes such as *erm*, *msr*, and *mef* (23). Hence, the need for the rapid and reliable identification of these genes and the investigation of the relationship with susceptible patterns has become more important (17). We detected *mefA*, *msrA*, and main *erm* genes using PCR with primers specific for the genes responsible for erythromycin resistance. It was found that neither *mefA* and *msrA* was detected among the resistant isolates, which further supported the evidence that efflux mechanism does not exist in the resistant isolates. To our knowledge, there have been no previous studies for the detection of *ermA* in *S. aureus* veterinary clinical isolates in China. In our results, no PCR products of erythromycin-resistant gene *ermA* was observed. However, the *ermA* was detected in all erythromycin-resistant MRSA (meticillin-resistant *S. aureus*) isolates from human clinical hospitals (17). This finding was also shown by Sekiguchi *et al.* (36) who reported that *ermA* is predominantly found in *S. aureus* isolates. The discrepancy between our findings and previous studies need to be further investigated. On the other hand the incidence of resistance genes, *ermB* genes were determined in 100% (26/26) of examined isolates and in 100% (20/20) of resistant isolates. The other resistant gene, *ermC* gene, was detected in 84.6% (22/26) of tested isolates and in 100% (20/20) of resistant isolates. Both genes were detected together in 22 of the resistant isolates (84.6%). In conformity to these results, the *ermB* gene was encountered more frequently among 26 isolates and the two genes exhibited same incidence in the resistant isolates. Wang *et al.* (8) found that the *ermB* gene was found mainly in cML isolates (87.5%), while the *ermC* gene was more common in isolates with iML phenotype (100%). Ardic *et al.* (29) reported that the detection rates of *ermC* genes were 64.3% in 56 methicillin-resistant *Staphylococcal* iso-

lates. These findings were similar to our findings, except for the determination of the *ermC* gene.

To date, there has been no previous report on the susceptible isolates containing resistant genes. Interestingly, in present study 6 erythromycin-susceptible isolates (*HLJ23-1*, *HLJ4*, *HLJ3*, *SH-11*, *SH-12*, and *SH-18*, MICs ≤ 0.151 μg/mL) were presented in *ermB*-carrying isolates, and 2 susceptible isolates (*HLJ23-1* and *HLJ4*) were detected in both *ermB*- and *ermC*-carrying isolates. Sequence analysis detected only one mutation at position 27 (A→T) in the region of *ermB* promotor of *SH-11* isolate, whereas two mutations occurred at position 115 (A→G) and 215 (A→G) in the region of leader peptide of *HLJ23-1* isolate, which demonstrated that much lower base mutations occurred in the non-structural region. In order to investigate whether the mutation in structural gene of *erm* plays a crucial role in erythromycin susceptibility, *erm* genes were cloned into erythromycin-susceptible *Transt-t1* bacterium. MICs of transformed bacteria significantly increased to 200 μg/mL, which proved that transformed bacteria had acquired stronger resistant characteristics. This evidence proved that slight mutations of *ermB* and *ermC* genes in the structural region did not affect the activity of methylase. On the basis of the above findings, we proposed that the mutation of 23S-*rRNA* may play a significant role in erythromycin susceptibility. A majority change of (G) mainly occurred in 23S-*rRNA* sequence, in accordance with the mutation of the resistant gene. Each isolate at least contained one base change. The methylation site occurred at position 2058 of 23S-*rRNA* (15). The conformational changes which occurred in the P site of ribosome protein prevented macrolide binding (9). However, the detected mutation at position 2057 (AG) of 23S-*rRNA* in *HLJ3* isolate which is near the position 2058 may influence methylation of P site and result in the rebinding of erythromycin. Similarly, Douthwaite (37) reported that three base substitutions at position 2032 of 23S-*rRNA* produce an erythromycin-hypersensitive phenotype. From this, we presumed that susceptible isolates were possibly engendered by the alteration of 23S-*rRNA* structure leading to the inability of the methylation, which could not prevent the rebinding of erythromycin. The evidence sufficiently illustrated that ribosome methylases encoded by *ermB* and *ermC* genes played a vital role in the resistance of *S. aureus* from bovine mastitis in China.

In conclusion, our results demonstrated a high proportion of inducible erythromycin-resistant *S. aureus* from clini-



cal bovine mastitis in China. Erythromycin resistance was caused mainly by methylation of *23S-rRNA* encoding by *ermB* and *ermC* genes. In this study we initially found that the mutation of *23S-rRNA* gave rise to many erythromycin-susceptible isolates by possibly disturbing the site of the methylation, allowing erythromycin to bind to the ribosome. The exact mutation site and its mechanism need to be further investigated.

### Conflict of interest statement

All authors declare that they do not have any financial or personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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