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Antimicrobial resistance and virulence genes of *Streptococcus agalactiae* isolated from mastitis milk samples in China

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Abstract

Introduction: *Streptococcus agalactiae* is an important zoonotic pathogen that affects milk production and quality and poses a threat to public health. Treatment of infections with this bacterium exploits antimicrobials, to which the resistance of *S. agalactiae* is a growing problem. Addressing the possibility of a correlation between this pathogen's genetic factors for antimicrobial resistance and virulence, this study attempted to identify the relevant genes. **Material and Methods:** Antimicrobial resistance of *S. agalactiae* isolated from 497 Chinese bovine mastitic milk samples was detected by the broth microdilution method. Eight drug resistance genes and eleven virulence genes were detected using PCR. **Results:** *Streptococcus agalactiae* was 100% susceptible to rifampicin and vancomycin, 93.33% susceptible to sulfisoxazole and sulfamethoxazole, but 100% resistant to ≥3 of the 16 antimicrobial agents, thereby being multidrug resistant, with resistance to oxacillin, tetracycline, erythromycin, clindamycin, and gentamicin being common. The *ermB*, *ermA* and *lnuA* genes were carried by 73.33%, 66.67% and 60.00% of the strains, respectively. The carriage rates of the *glnA, clyE, hylB*, *bibA*, *iagA*, and *fbsA* virulence genes were greater than 40%, *lmb* and *bac* were not observed in any strain, and *glnA+hylB+bibA+iagA+fbsA+clyE* combined virulence gene patterns were the most commonly detected. **Conclusion:** Antimicrobial resistance of *S. agalactiae* is still a great concern for cattle health in China, and multidrug resistance coupled with the high positive rates of this bacterium's strains for virulence genes indicates the importance of *S. agalactiae* surveillance and susceptibility tests.

Keywords: cattle, bovine mastitis, dairy industry, antimicrobial stewardship, virulence gene.

Introduction

Streptococcus agalactiae, or group B *Streptococcus* (GBS), is a Gram-positive, facultatively anaerobic bacterium which was first described as the leading causative pathogenic bacteria of bovine mastitis (45). It causes a decline in milk production and quality, serious economic losses, and poses a substantial challenge to public health, making it a key challenge for the dairy industry. Antibiotic treatment is the first line of defence against mastitis (38); however, antimicrobial resistance and antimicrobial residues are areas of increasingly serious concern in both human and veterinary medicine (37). This coccus uses virulence factors encoded by its genes to enter, replicate and persist in the host (17). Antimicrobial resistance genes confer GBS with the ability to resist the effects of antibiotic medication, and these genes are found in the environment (27). Therefore, resistance and virulence are two key factors that determine the degree of infection; they may be correlated with each other (14). There are a variety of pathogenicity-related virulence factors associated with *S.*

agalactiae, including adhesion-, invasion-, and immune escape-related factors. The adhesion-related virulence genes include *fbsA*, *scpB* and *lmb,* which encode fibrinogen binding protein A, C5a peptidase, and laminin binding protein, respectively (7, 32). Invasion-related virulence genes include *cylE*, *cfb*, *hylB* and *bca*, which encode β-haemolysin, CAMP factor and α-protein, respectively, as well as *hylB*, which participates in the formation of hyaluronate lyase (12, 24). Some virulence genes, such as *bac,* facilitate immune evasion (16). Strongly drug-resistant strains were found to carry few virulence genes, confirming that resistance and virulence were negatively correlated (35). A contrasting positive correlation was posited by Arshadi *et al.* (2), who showed that virulence gene–positive strains became more pathogenic and more difficult to eliminate as drug resistance increased. The inexistence of any correlation is also possible, because one study reported drug resistance which was not related to virulence (36).

Conducting antimicrobial resistance studies is imperative for selecting the most appropriate antimicrobial therapies and reducing the risk of further development and spread of antimicrobial resistance through the horizontal transfer of resistance genes. Investigations of the *S. agalactiae* virulence and antibiotic resistance genes are important for the development of vaccines and for understanding the resistance mechanisms used by pathogens. The objective of this study was to identify the virulence and antibiotic resistance genes found in *S. agalactiae* isolates from mastitis milk samples in China, as well as to study phenotypic antimicrobial resistance and the correlation between the genes of one type and those of the other.

Material and Methods

Bacterial strains. Fifteen *S. agalactiae* strains were isolated from 497 bovine mastitis milk samples at the Agricultural Quality and Safety Laboratory of Xinjiang according to the NY/T 2962-2016 standard (42). Standard strains of *S. agalactiae* (American Type Culture Collection (ATCC)12386) were purchased from BaoxinBio Inc. (Urumqi, China).

Antimicrobial susceptibility tests. The broth dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI) (9) was used to evaluate the minimum inhibitory concentrations (MICs) of 16 commonly used antimicrobial drugs to suppress growth of the 15 *S. agalactiae* strains (Table 1). Each antibiotic tested was serially twofold diluted and wells containing different concentrations were prepared: penicillin (PEN), ampicillin (AMP), clindamycin (CLI), ciprofloxacin (CIP), and rifampicin (RIF) were prepared at concentrations of 64.0, 32.0, 16.0, 8.0, 4.0, 2.0, 1.0, 0.5, 0.25 and 0.125 mg/mL; erythromycin (ERY), cefoxitin (CET), oxacillin (OXA), tetracycline (TE), doxycycline (DOX), gentamicin (GM), florfenicol (FFC), and vancomycin (VAN) were prepared at concentrations of 128.0, 64.0, 32.0, 16.0, 8.0, 4.0, 2.0, 1.0, 0.5, 0.25 and 0.125 mg/mL; sulfisoxazole (SMZ) was prepared at concentrations of 1024, 512, 256, 128, 64, 32, 16, 8, 4 and 2 mg/mL; amoxicillin/clavulanic acid (A/C) was prepared at concentrations of 128/64, 64/32, 32/16, 16/8, 8/4, 4/2, 2/1, 1/0.5, 0.5/0.25 and 0.25/0.12 mg/mL; and sulfamethoxazole (SXT) was prepared at concentrations of 64/1208, 32/604, 16/304, 8/152, 4/76, 2/38, 1/19, 0.5/9.5, 0.25/4.8 and 0.12/2.4 mg/mL. *Streptococcus agalactiae* strains (ATCC12386) were used as a quality control in this study. Because no specific resistance breakpoints for *Streptococcus* spp. were available for some tested antimicrobials, the resistance breakpoints for an antimicrobial of the same class were referred to as recommended by the CLSI (9).

Table 1. 16 kinds of antibacterial drugs and their classification

Drug category	Antibacterial drugs
β-lactams	penicillin
	ampicillin
	amoxicillin / clavulanic acid
	oxacillin
	cefoxitin
Macrolides	erythromycin
Lincosamides	clindamycin
Aminoglycosides	gentamicin
Tetracyclines	doxycycline
	tetracycline
Chloramphenicols	florfenicol
Ansamycins	rifampicin
Glycopeptides	vancomycin
Quinolones	ciprofloxacin
Sulfonamides	sulfisoxazole
	sulfamethoxazole

Genomic DNA extraction. The strains were inoculated into BHI broth and cultured for 18–24 h at 36°C, after which genomic DNA was extracted according to the Bacterial DNA Extraction kit manufacturer's instructions (Tiangen BioTech, Beijing, China). The concentration and mass of extracted DNA were determined using a Thermo NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). All DNA samples were stored at −20°C until use.

PCR amplification of drug resistance genes and virulence genes. The primer sequences for the drug resistance and virulence genes of *S. agalactiae* were synthesised at the Beijing Genome Institute (Beijing, China) (Table 1). The PCR amplification reaction was performed in a 25 μL mixture volume comprised of 12.5 μL of 2 × Taq PCR Master Mix enzyme (Takara BioTech, Kusatsu, Japan), 1 μL of upstream and downstream primers, 2 μL of DNA template and 8.5 μL of sterilised ultrapure water. The programme for amplification of resistance genes was as follows: pre-denaturation at 94°C for 4 min, denaturation at 94°C for 30 s, annealing at a temperature determined individually for each gene (Table 2), and extension at 72°C for 90 s. Thirty cycles were performed, followed by incubation at 72°C for 10 min.

Table 2. Primer sequence and reaction conditions of the drug resistance gene of *S. agalactiae*

Antimicrobial drug class	Resistance gene	Primer sequence (5′–3′)	Annealing temperature (°C)	PCR product size (bp)	Reference
Lincosamides	*lnuA*	F: GGTGGCTGGGGGGTAGATGTATTAACTGG R: GCTCTCTTTGAAATACATGGTATTTTTCGATC	56	323	(23)
Tetracyclines	*tetO*	F: AACTTAGGCATTCTGGCTCAC R: TCCCACTGTTCCATATCGTCA	50	515	(29)
	tetK	F: TCGATAGGAACAGCAGTA R: CAGCAGATCCTACTCCTT	44	169	(33)
	tetM	F: GTGGACAAAGGTACAACGAG R: CGGTAAAGTTCGTCACACAC	50	406	(39)
	tetS	F: CATAGACAAGCCGTTGACC R: ATGTTTTTGGAACGCCAGAG	48	667	
Macrolides	*ermB*	F: CGAGTGAAAAAGTACTCAACC R: AGTAACGGTACTTAAATTGTTTAC	48	652	(32)
	ermC	F: ATCTTTGAAATCGGCTCAGG R: CAAACCCGTATTCCACGATT	47	295	
	ermA	F: GTTCAAGAACAATCAATACAGAG R: GGATCAGGAAAAGGACATTTTAC	48	421	

F – forward; R – reverse

Table 3. Primer sequence and reaction conditions of the virulence gene of *S. agalactiae* (13, 22, 23)

Virulence factor	Virulence gene	Primer sequence (5′–3′)	Annealing temperature (°C)	PCR product size (bp)
β-haemolysin/cytolysis	*cylE*	F: CATTGCGTAGTCACCTCCC R: GGGTTTCCACAGTTGCTTGA	54	399
αC protein	*Bca*	F: TAACAGTTATGATACTTCACAGAC□ R: ACGACTTTCTTCCGTCCACTTAGG	51	535
C5a peptidase	*scpB*	F: CCAAGACTTCAGCCACAAGG R: CAATTCCAGCCAATAGCAGC	57	591
Laminin binding protein	*lmb*	F: ACCGTCTGAAATGATGTGG R: GATTGACGTTGTCTTCTGC	51	572
Glutamine synthetase	*glnA*	F: ACGTATGAACAGAGTTGGCTATAA R: TCCTCTGATAATTGCATTCCAC	52	471
CAMP factor	*Cfb*	F: ATGGGATTTGGGATAACTAAGCTAG R: AGCGTGTATTCCAGATTTCCTTAT	52	193
Hyaluronidase	*hylB*	F: ACAAATGGAACGACGTGACTAT R: CACCAATTGGCAGAGCCT	52	346
βC protein	*bac*	F: AAGCAACTAGAAGAGGAAGC R: TTCTGCTCTGGTGTTTTAGG	53	479
Bacterial immunogenic adhesive	*bibA*	F: AACCAGAAGCCAAGCCAGCAACC R: AGTGGACTTGCGGCTTCACCC	58	127
Invasion-associated gene	*iagA*	F: CGGGATTGATCTAAGTCGCT R: CCATCAACATCAGTCGCTAA	53	459
Fibrin binding protein B	*fbsA*	F: AGAGCCAAGTAGGTCAACTTATAG R: TTCATTGCGTCTCAAACCG	54	290

F – forward; R – reverse; CAMP – Christie–Atkins–Munch-Peterson

The program for amplification of the virulence gene was pre-denaturation at 95°C for 5 min, followed (as in the reaction for resistance genes) by denaturation at 94°C for 30 s, annealing at a temperature determined individually for each gene (Table 3), and extension at 72°C for 1 min. As previously, thirty cycles were performed, followed by incubation at 72°C for 10 min. The amplified products were electrophoresed on a 1% agarose gel with 1× tris-acetate-ethylenediaminetetraacetic acid buffer. Electrophoresis was performed at 120 V for 30 min with gel pictures taken afterwards using a Bio-Rad Gel Doc XR molecular imaging system (Bio-Rad, Hercules, CA, USA).

Gene sequencing and analysis. The PCR products and upstream and downstream primers of each gene were sent to Sangon Biotech Co. Ltd. (Shanghai, China) for sequencing. Homology for the obtained DNA sequence was then searched for in NCBI databases (www.ncbi.nlm.nih.gov/) to determine the genotype.

Correlation between phenotype and genotype of *S. agalactiae.* The consistency rate between the genotype and phenotype of the drug resistance of *S. agalactiae* to the 16 antibiotics was determined according to the positivity rate of the resistance genotype and the resistance phenotype of *S. agalactiae* defined as follows:

$$\text{Drug resistance rate} = \frac{\text{Number of multidrug – resistant bacteria}}{\text{Total number of pathogen strains}} \times 100\%$$

$$\text{Phenotype resistance rate} = \frac{\text{Number of resistance rates of similar drug strains}}{\text{Number of similar drugs}} \times 100\%$$

$$\text{Consistency rate} = \frac{\text{(related drug resistance gene strains)positive rate}}{\text{phenotypic resistance rate}} \times 100\%$$

Resistance to three or more antibiotics is the definition for a strain to be designated multi drug resistant (MDR). The result interpretation was performed according to the CLSI guidelines (9).

Statistical analysis. All data entry and analyses were performed using the Statistical Package for the Social Sciences (SPSS) software, version 24.0 (IBM, Armonk, NY, USA). Binary logistic regression was used to analyse the relationship between antibiotics and virulence genes, the antibiotic resistance was adopted as the dependent variable and the detection of virulence genes as the independent variable to be included in the binary logistic regression model. The cut-off for statistical significance was set at $P < 0.05$.

Results

Results of the antibiotic sensitivity test of *S. agalactiae* isolates. Susceptibility testing of 16 antibiotics on 15 *S. agalactiae* strains (Table 4) showed that collectively they were 100% (n = 15) susceptible to RIF and VA, highly susceptible to SMZ and SXT (93.33%, n = 14), and moderately susceptible to A/C, AMP, CET, DOX, and CIP (all >70.00%, n = 10 and n = 11). The strains were highly resistant to OXA, TE, and ERY (80.00%, n = 12), and moderately resistant to CLI (66.67%, n = 10). The drug classes which encountered the highest resistance were macrolides (80.00%), lincosamides (66.67%), and tetracyclines (46.67%). The lowest and highest MIC values were 0.5 and 32 μg/mL, respectively for CET, 0.5 and 32 μg/mL for OXA, 2 and 256 μg/mL for SMZ, 4 and 64 μg/mL for TE, 0.5 and 32 μg/mL for DOX, 0.5 and 64 μg/mL for ERY, 0.25 and 16 μg/mL for CLI, 1 and 16 μg/mL for GM, 0.5 and 4 μg/mL for CIP and 2 and 16 μg/mL for FFC.

Multidrug resistance pattern. Analysis of *S. agalactiae* showed that 100% of the strains were resistant to more than three antimicrobial agents. The major MDR profile observed in the multidrug-resistant isolates was OXA-TE-ERY-CLI-GM. Four isolates (26.67%) were resistant to three antimicrobials, one isolate (6.67%) was resistant to four of the preparations, five isolates (33.33%) were resistant to five of them, one isolate (6.67%) was resistant to six, three isolates (20.00%) were resistant to seven, and one isolate (6.67%) was resistant to nine antimicrobials. Therefore, resistance to three and five antibiotics was the most common, and resistance to up to nine antibiotics was observed (Fig. 1).

Consistency between resistance genotype and phenotype of *S. agalactiae.* Table 5 shows the occurrence of different resistance genes among the *S. agalactiae* isolates. Of the eight resistance genes screened for, *ermB* (n = 11) was detected as the most prevalent by PCR, followed by *ermA* (n = 10), and *lnuA* (n = 9).

The strains' positivity rates for *ermB*, *ermA* and *ermC* were 73.33%, 66.67%, and 33.33%, respectively. Of the 12 isolates that demonstrated resistance to macrolide antibiotics, 11 (91.66%) carried *ermB*, 10 (83.33%) *ermA* and 5 (41.66%) *ermC*. The prevalence rate of *lnuA* was 60%. Isolates demonstrating resistance to lincosamide antibiotics, of which there were 10, were in 9 instances (89.99%) positive for *lnuA* genes. The detection rates for *tetM*, *tetK*, *tetS* and *tetO* were 46.67%, 40.00%, 40.00% and 33.33%, respectively. Overall, we found three resistance genes encoding macrolide resistance (*ermB*, *ermA* and *ermC*), one gene for lincosamide resistance (*lnuA*), and four genes (*tetM*, *tetK*, *tetS* and *tetO*) for tetracycline resistance.

Distribution of virulence genes of *S. agalactiae.* Fifteen isolates were screened for 12 genes potentially involved in virulence using PCR. The *lmb* and *bac* genes were not present in *S. agalactiae* isolates, while the *fbsA* gene was harboured by 14 isolates (93.33%). The *clyE*, *hylB*, *bibA* and *iagA* genes were discovered in *S. agalactiae* at incidences of 53.33%, 80.00%, 73.33%, and 86.67%, respectively. In contrast, *glnA*, *bca*, *cfb* and *scpB* were identified in only six (40.00%), four (26.67%), three (20.00%), and one (6.67%) of the isolates, respectively (Fig. 2). In relation to the virulence genes screened for in this study, eleven virulence profiles were detected, which were *glnA+hylB+bibA+iagA+fbsA+clyE*, *glnA+hylB+iagA+fbsA*, *bca+glnA+hylB+iagA+fbsA+clyE*, *bca+cfb+hylB+bibA+iagA+fbsA+clyE*, *bibA+iagA+fbsA+clyE*, *bca+hylB+bibA+iagA+fbsA*, *hylB+bibA+iagA+fbsA*, *bca+bibA+iagA+fbsA*, *hylB+bibA+iagA+fbsA+clyE*, *cfb+hylB+bibA+iagA+fbsA* and *cfb+hylB+bibA+fbsA+clyE* (Table 6). The raw mastitic milk samples contained *S. agalactiae* strains with *glnA+hylB+bibA+iagA+fbsA+clyE* and *glnA+hylB+iagA+fbsA* as the virulence gene combinations most commonly detected.

Correlation between drug resistance and virulence genes in multidrug resistant *S. agalactiae.* The multiple-drug-resistant strains carried different virulence genes (Table 7). However, analysis of drug resistance and virulence genes of multidrug-resistant *S. agalactiae* showed that all such strains carrying the *hylB*, *iagA* and *fbsA* virulence genes, as well all of as those carrying the *ermB* and *lnuA* resistance genes, were resistant to both tetracycline (90%) and clindamycin (70%) (Table 6). However, there was no significant correlation between virulence genes and the MDR of *S. agalactiae* strains ($P > 0.05$) (Table 8).

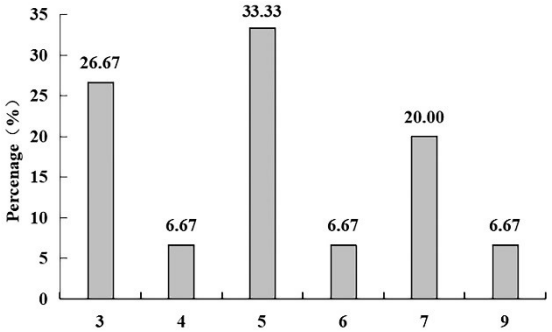

Fig. 1. Multiple drug resistance of *S. agalactiae* shown as antibiotic phenotypic resistance and resistance gene prevalence data. 3 – resistance to 3 classes of antimicrobials; 4 – resistance to 4 classes of antimicrobials; 5 – resistance to 5 classes of antimicrobials; 6 – resistance to 6 classes of antimicrobials; 7 – resistance to 7 classes of antimicrobials; 9 – resistance to 9 classes of antimicrobials

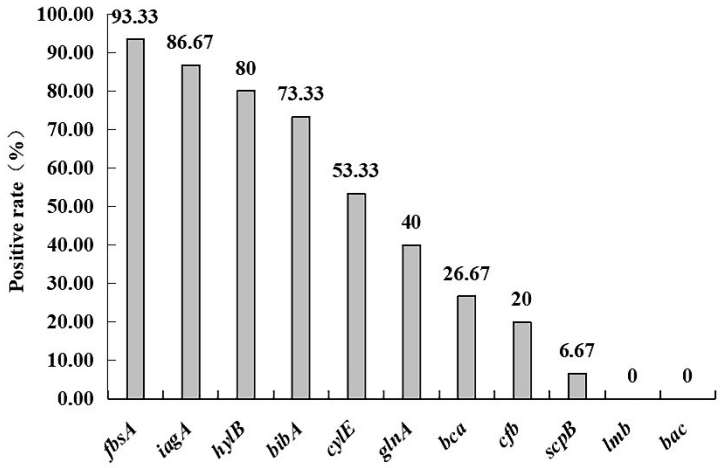

Fig. 2. Positive rate of *S. agalactiae* resistance genes

Table 4a. Minimum inhibitory concentration (MIC) values of, and resistance of *S. agalactiae* isolates to, β-lactams, sulfonamides and tetracyclines

Isolate no.	MIC (μg/mL)								
	PEN	A/C	AMP	CET	OXA	SMZ	SXT	TE	DOX
SH07	≤0.125 (S[3])	1/0.5 (R)	≤0.125 (S)	32 (R)	1 (S)	4 (S)	0.5/9.5 (S)	4 (S)	4 (S)
SH07-2	4 (R)	≤0.25/0.12 (S)	≤0.125 (S)	0.5 (S)	16 (R)	256 (I)	0.25/4.8 (S)	16 (R)	2 (S)
SH12	≤0.125 (S)	≤0.25/0.12 (S)	0.25 (I)	0.5 (S)	4 (R)	8 (S)	0.5/9.5 (S)	32 (R)	4 (S)
SH33	≤0.125 (S)	≤0.25/0.12 (S)	≤0.125 (S)	0.5 (S)	16 (R)	64 (S)	0.5/9.5 (S)	32 (R)	0.5 (S)
SH45	≤0.125 (S)	≤0.25/0.12 (S)	≤0.125 (S)	1 (S)	32 (R)	64 (S)	0.25/4.8 (S)	64 (R)	2 (S)
HLJ008	≤0.125 (S)	≤0.25/0.12 (S)	≤0.125 (S)	32 (R)	32 (R)	128 (S)	0.25/4.8 (S)	32 (R)	2 (S)
HLJ048-2	2 (R)	≤0.25/0.12 (S)	≤0.125 (S)	1 (S)	4 (R)	2 (S)	0.5/9.5 (S)	16 (R)	8 (I)
HLJ030-3	≤0.125 (S)	≤0.25/0.12 (S)	≤0.125 (S)	0.5 (S)	0.5 (S)	64 (S)	0.5/9.5 (S)	64 (R)	4 (S)
NM025	≤0.125 (S)	≤0.25/0.12 (S)	≤0.125 (S)	1 (S)	1 (S)	64 (S)	0.25/4.8 (S)	32 (R)	16 (R)
HB016	≤0.125 (S)	≤0.25/0.12 (S)	≤0.125 (S)	0.25 (S)	16 (R)	128 (S)	0.5/9.5 (S)	16 (R)	4 (S)
NM-2-72-4	8 (R)	≤0.25/0.12 (S)	2 (R)	1 (S)	16 (R)	128 (S)	0.25/4.8 (S)	32 (R)	32 (R)
NM-2-034-3	8 (R)	16/8 (R)	4 (R)	0.5 (S)	4 (R)	64 (S)	2/38 (I)	4 (S)	4 (S)
SH-2-46-1	≤0.125 (S)	≤0.25/0.12 (S)	≤0.125 (S)	0.5 (S)	32 (R)	128 (S)	0.25/4.8 (S)	16 (R)	4 (S)
SH-2-14-2	2 (R)	4/2 (R)	≤0.125 (S)	1 (S)	16 (R)	128 (S)	0.25/4.8 (S)	32 (R)	8 (I)
SD-2-009-2	≤0.125 (S)	16/8 (R)	≤0.125 (S)	0.5 (S)	4 (R)	128 (S)	0.25/4.8 (S)	8 (I)	4 (S)
	Drug resistance rate (%) (/15)								
S	66.67 (10)	73.33 (11)	80.00 (12)	86.67 (13)	20.00 (3)	93.33 (14)	93.33 (14)	13.33 (2)	73.33 (11)
I	0 (0)	0 (0)	6.67 (1)	0 (0)	0 (0)	6.67 (1)	6.67 (1)	6.67 (1)	13.33 (2)
R	33.33 (5)	26.67 (4)	13.33 (2)	13.33 (2)	80.00 (12)	0 (0)	0 (0)	80.00 (12)	13.33 (2)

PEN – penicillin; A/C – amoxicillin/clavulanic acid; AMP – ampicillin; CET – cefoxitin; OXA – oxacillin; SMZ – sulfisoxazole; SXT – sulfamethoxazole; TE – tetracycline; DOX – doxycycline

Table 4b. Minimum inhibitory concentration (MIC) values of, and resistance of *S. agalactiae* isolates to, a macrolide, a lincosamide, an aminoglycoside, a quinolone, a chloramphenicol, an ansamycin, and a glycopeptide

Isolate no.	MIC (μg/mL)						
	ERY	CLI	GM	CIP	FFC	RIF	VAN
SH07	16 (R)	0.25 (S)	4 (S)	0.5 (S)	2 (S)	0.5 (S)	0.5 (S)
SH07-2	16 (R)	4 (R)	4 (S)	0.5 (S)	16 (R)	0.125 (S)	0.5 (S)
SH12	32 (R)	8 (R)	16 (R)	0.5 (S)	4 (S)	0.5 (S)	0.5 (S)
SH33	16 (R)	8 (R)	16 (R)	0.5 (S)	8 (I)	0.5 (S)	0.5 (S)
SH45	64 (R)	8 (R)	2 (S)	2 (I)	8 (I)	0.5 (S)	0.5 (S)
HLJ008	16 (R)	4 (R)	4 (S)	0.5 (S)	4 (S)	0.5 (S)	0.5 (S)
HLJ048-2	16 (R)	16 (R)	8 (I)	0.5 (S)	4 (S)	1 (S)	0.5 (S)
HLJ030-3	0.5 (S)	16 (R)	2 (S)	0.5 (S)	16 (R)	0.5 (S)	0.5 (S)
NM025	16 (R)	0.5 (S)	1 (S)	0.5 (S)	8 (I)	0.5 (S)	0.5 (S)
HB016	16 (R)	4 (R)	16 (R)	0.5 (S)	4 (S)	0.5 (S)	0.5 (S)
NM-2-72-4	2 (I)	0.5 (S)	32 (R)	0.5 (S)	16 (R)	0.5 (S)	0.5 (S)
NM-2-034-3	0.5 (S)	16 (R)	4 (S)	4 (R)	16 (R)	0.5 (S)	0.5 (S)
SH-2-46-1	16 (R)	0.5 (S)	4 (S)	0.5 (S)	4 (S)	0.5 (S)	0.5 (S)
SH-2-14-2	16 (R)	16 (R)	4 (S)	4 (R)	8 (I)	0.5 (S)	0.5 (S)
SD-2-009-2	32 (R)	0.5 (S)	16 (R)	4 (R)	16 (R)	0.5 (S)	0.5 (S)
	Drug resistance rate (%) (/15)						
S	13.33 (2)	33.33 (5)	60.00 (9)	73.33 (11)	40.00 (6)	100 (15)	100 (15)
I	6.67 (1)	0 (0)	6.67 (1)	6.67 (1)	26.67 (4)	0 (0)	0 (0)
R	80.00 (12)	66.67 (10)	33.33 (5)	20.00 (3)	33.33 (5)	0 (0)	0 (0)

ERY – erythromycin; CLI – clindamycin; GM – gentamycin; CIP – ciprofloxacin; FFC – florfenicol; RIF – rifampicin; VAN – vancomycin

Table 5. Relationship between drug resistance phenotype and drug resistance genotype of *S. agalactiae*

Antibiotic type	Resistance gene	Genotype (%)		Phenotype resistance rate (%)	Consistency rate (%)
		Number of bacteria	Positive rate		
Macrolides	*ermB*	11/15	73.33	80.00	91.66
	ermA	10/15	66.67		83.33
	ermC	5/15	33.33		41.66
Lincosamides	*lnuA*	9/15	60.00	66.67	89.99
Tetracyclines	*tetM*	7/15	46.67	46.67	100.00
	tetK	6/15	40.00		85.71
	tetS	6/15	40.00		85.71
	tetO	5/15	33.33		71.42

Table 6. The combined virulence gene profile of *S. agalactiae*

Virulence gene profile	Distribution[a] (%)
glnA+hylB+bibA+iagA+fbsA+clyE	3 (20)
glnA+hylB+iagA+fbsA	2 (13.33)
bca+glnA+hylB+iagA+fbsA+clyE	1 (6.67)
bca+cfb+hylB+bibA+iagA+fbsA+clyE	1 (6.67)
bibA+iagA+fbsA+clyE	1 (6.67)
bca+hylB+bibA+iagA+fbsA	1 (6.67)
hylB+bibA+iagA+fbsA	1 (6.67)
bca+bibA+iagA+fbsA	1 (6.67)
hylB+bibA+iagA+fbsA+clyE	1 (6.67)
cfb+hylB+bibA+iagA+fbsA	1 (6.67)
cfb+hylB+bibA+fbsA+clyE	1 (6.67)

[a] – Distribution was achieved based on the total numbers of 15 *S. agalactiae* isolates

Table 7. The drug resistance patterns and gene expression profiles of *S. agalactiae*

Isolate no.	Drug resistance pattern	Resistant gene	Virulence gene
SH07	(A/C)/CET/ERY	*ermB+lnuA+tetM+tetO*	*bca+hylB+bibA+iagA+fbsA*
SH07-2	PEN/OXA/TE/ERY/CLI/FFC	*ermA+ermB+lnuA+tetM+tetK+tetS+tetO*	*glnA+hylB+iagA+fbsA*
SH12	OXA/TE/ERY/CLI/GM	*ermA+ermB+ermC+lnuA+tetS+tetO*	*bca+glnA+hylB+iagA+fbsA+clyE*
SH33	OXA/TE/ERY/CLI/GM	*ermA+ermB+ermC+lnuA+tetK+tetS+tetO*	*bca+cfb+hylB+bibA+iagA+fbsA+clyE*
SH45	OXA/TE/ERY/CLI	*ermA+ermB+lnuA+tetK+tetS*	*hylB+bibA+iagA+fbsA*
HLJ008	CET/OXA/TE/ERY/CLI	*ermB+ermA+ermC+lnuA+tetM+tetK*	*glnA+hylB+bibA+iagA+fbsA+clyE*
HLJ030-3	TE/CLI/FFC	*ermB+ermA+lnuA+tetM*	*hylB+bibA+iagA+ fbsA+clyE*
NM025	TE/DOX/ERY	*ermA+ermB+lnuA+tetK+tetO*	*glnA+hylB+bibA+iagA+fbsA+clyE*
HB016	OXA/TE/ERY/CLI/GM	*ermA+ermB+ermC+lnuA+tetM+tetK+tetS*	*glnA+hylB+bibA+iagA+fbsA+clyE*
SH-2-46-1	OXA/TE/ERY	*ermA+ermB+lnuA+tetO*	*glnA+hylB+iagA+fbsA*

A/C – amoxicillin/clavulanic acid; CET – cefoxitin; CLI – clindamycin; ERY – erythromycin; FFC – florfenicol; GM – gentamicin; OXA – oxacillin; PEN – penicillin; TE – tetracycline

Table 8. Logistic regression analysis of the relationship between antibiotics and virulence genes

Virulence gene	Penicillin			Cefoxitin			Oxacillin			Doxycycline			Gentamicin			Clindamycin		
	B	Exp(B)	P	B	Exp(B)	P	B	Exp(B)	P	B	Exp(B)	P	B	Exp(B)	P	B	Exp(B)	P
bca	40.537	4.028	0.999	1.099	3.000	1.000	−42.406	0.000	0.999	1.099	3.000	1.000	−41.307	0.000	0.999	−61.799	0.000	0.999
scpB	−20.269	0.000	1.000	−60.718	0.000	0.999	62.916	2.108	0.999	19.699	3.590	1.000	62.105	9.369	0.999	−62.493	0.000	0.999
glnA	0.000	1.000	1.000	−19.411	0.000	0.999	−21.896	0.000	0.999	−19.411	0.000	0.999	−19.411	0.000	0.999	−0.693	0.500	0.744
cfb	−0.934	0.393	1.000	42.406	2.610	0.999	−84.812	0.000	0.999	−38.011	0.000	1.000	−80.417	0.000	0.999	−81.880	0.000	0.999
hylB	42.406	2.610	0.999	−40.209	0.000	0.999	42.406	2.610	0.999	40.209	2.900	0.999	40.209	2.900	0.999	0.000	1.000	1.000
bibA	21.203	1.615	1.000	−19.411	0.000	1.000	−21.896	0.000	1.000	−19.411	0.000	1.000	−19.411	0.000	1.000	−42.412	0.000	0.999
iagA	−41.472	0.000	1.000	−1.099	0.333	1.000	42.406	2.610	1.000	−1.099	0.333	1.000	41.307	8.699	1.000	−20.080	0.000	1.000
cylE	0.000	1.000	1.000	−1.099	0.333	1.000	42.406	2.610	0.999	−1.099	0.333	1.000	−1.099	0.333	1.000	21.203	1.615	1.000

Exp (B) – OR value, logarithmic ratio, equal to the exponential power of the regression coefficient

Discussion

Bovine mastitis is often caused by *S. agalactiae*, which is a highly contagious obligate bacterial pathogen of the mammary gland resulting in milk contamination and causing potential harm to human health (20, 22). Contributions to the literature on *S. agalactiae* as the aetiological agent of bovine mastitis originate from many parts of the world: previous studies have shown that bovine mastitis outbreaks caused by *S. agalactiae*

occurred more frequently in Denmark and Norway than elsewhere (1, 20) and that it is also the most common pathogen causing bovine mastitis in China (47).

Antibiotics remain the preferred treatment for bovine mastitis. However, in recent years, with the improper use of antibiotics, the problems of drug residues and resistance have become increasingly serious, not only threatening human health, but also bringing new challenges to the treatment of bovine mastitis. This study showed that *S. agalactiae* is highly resistant to antibiotics, particularly macrolides (80.00%), lincosamides (66.67%) and tetracycline (46.67%) and highly susceptible to ansamycins (100%), glycopeptides (100%), sulfonamides (93.33%) and quinolones (73.33%). We also showed that 100% of strains exhibited MDR, revealing the severity of this problem in China.

Previous studies showed that *S. agalactiae* was highly resistant to β-lactams, macrolides, and lincosamides, and was highly sensitive to quinolones and tetracyclines. In Yunnan province, China, *S. agalactiae* was resistant to multiple drugs, including β-lactams, macrolides, and sulfonamides (40). In Jordan and Brazil, *S. agalactiae* was reported to be extensively resistant to aminoglycosides and tetracyclines (11). According to the World Organisation for Animal Health (43), tetracyclines and macrolides were the two classes of antibiotics most commonly used in animals worldwide between 2010 and 2015. In our study, the results of the antimicrobial resistance tests indicated that *S. agalactiae* isolates from cows differed in their antimicrobial susceptibility patterns. The isolates were resistant to tetracycline and erythromycin, which is consistent with previous reports on bovine *S. agalactiae* antibiotic resistance and provides further evidence that antibiotic-resistant *S. agalactiae* are a global problem. We strongly recommend that tetracycline and erythromycin should not be used in the treatment of dairy cow mastitis in China, owing to their ineffectiveness against the 80% of *S. agalactiae* strains which can resist them. This conclusion will be shared with local dairy farms as a guide for the accurate prevention and treatment of bovine mastitis caused by this pathogen.

In *Streptococcus*, resistance to tetracyclines is encoded by ribosome protection genes including *tetM* and *tetO* or by the *tetK* and *tetL* efflux pump genes (34). Resistance to macrolides is due to two common mechanisms: a ribosome methylase, encoded by the *erm* genes, and an active efflux pump by a membrane-bound protein encoded by the *mef* gene. Resistance to clindamycin is encoded by a lincosamide that inactivates the *lnuA* nucleotidyl transferase gene. The results of this study showed that the dominant drug resistance gene of *S. agalactiae* was *ermB* and that between the resistance phenotype and the carrier rate for macrolides, lincosamides, and tetracyclines there was substantial consistency with an average rate of >70%. The *ermB* and *ermA* genes were detected in 73.33% and 66.68% of the 15 *S. agalactiae* isolates, respectively, suggesting that erythromycin-resistant methylase may be the major mechanism of resistance in the present study. Similar findings were reported by Hernandez *et al*. (18) for *S. agalactiae* strains isolated from Argentinean cattle with mastitis and by da Silva *et al*. (10). The prevalence of the *ermB* determinant shows that *S. agalactiae* often uses a target methylation mechanism for macrolide resistance.

S. agalactiae exhibits resistance to antibiotics through various metabolic mechanisms mediated by the corresponding resistance genes. The *mefA* gene–mediated efflux pump mechanism is the main pathway of the bacterium's resistance to macrolides (5), *erm* leads to resistance to erythromycin by changing the drug target and methylation of the ribosomal 23S rRNA (41), and the *tet*-mediated ribosome protective protein mechanism confers resistance to tetracycline (4). This study suggests that *S. agalactiae* in China may have gained resistance to macrolides and tetracycline *via* ribosome binding protein sites and efflux pump mechanisms.

The PCR assay for the detection of virulence genes revealed that a high percentage of the *S. agalactiae* isolates were positive for *fbsA* (93.33%, 14/15), *iagA* (86.67%, 13/15) and *hylB* (80.00%, 13/15) (Fig. 2). The high prevalence of these genes in *S. agalactiae* has been reported previously (6, 30). However, higher frequencies were not universally detected for all virulence genes. We observed a low frequency of *scpB* (6.67%) and *bca* (26.67%) in *S. agalactiae* isolates, and obtained results inconsistent with the 90.1% for *scpB* and 86.0% for *bca* reported in Zimbabwe (18). Furthermore, none of the isolates in the present study possessed the *bac* or *lmb* genes. The *bac* gene was found with a similar frequency to that resulting from previous studies in the USA and Sweden with 20% and 12% of group B *Streptococcus* possessing *bac*, respectively (26, 31). A study conducted in Argentina in 2021 also observed high frequencies for *hylB* (100%) and *cpsA* (96%) and a lower frequency for *bca* (36%) in *S. agalactiae*; *bac*, *lmb*, and *scpB* genes could not be detected in any of the isolates (28). Our comparison with studies from Zimbabwe, the USA, Sweden, and Argentina suggests that discrepancies may be due to geographical location among other factors.

Research has found certain regional differences in the prevalence of *S. agalactiae* drug resistance phenotypes and virulence genes. Besides the relevance of this in bovine mastitis treatment, it is also an aspect to take into account in healthcare provision in humans. The results for the drug resistance and virulence of bovine *S. agalactiae* in our study were compared with those from analysis of strains isolated from human samples. Associated research in humans has often focused on *S. agalactiae* infection in pregnancy, because it can cause abortion and premature rupture of membranes and because when postpartum intrauterine infection with these bacteria becomes severe, it can lead to neonatal infection and other risks (8). Penicillin is often used

when *S. agalactiae* causes adverse reactions during pregnancy, but erythromycin and clindamycin are often used for treatment in patients with allergic reactions to penicillin, resulting in a gradual increase in the drug-resistance rate of human *S. agalactiae,* which is consistent with our study. According to research results on human *S. agalactiae* from Guangzhou (25), Urumqi (19), Shenzhen (48), Beijing (41), Taiwan and Zhejiang (46) in China, the *ermB* gene is the main mediating gene of *S. agalactiae* resistance to erythromycin from both human and bovine sources. However, there are some differences in the genes related to drug resistance in *S. agalactiae* isolated from different regions. In addition to *ermB*, *ermA*, *ermC*, and *ermTR*, there are other drug resistance genes. When we compared the virulence genes of *S. agalactiae* with those identified in studies on *S. agalactiae* isolated from people in Guangzhou (48) and Hainan (40), we found that they carried *cylE*, *hylB*, *bibA*, *iagA*, *bca*, *cfb* and *scpB*, but not *fbsA*, *cyl* or *glnA*. A possible reason for this may be that different virulence genes are carried in different regions and sources.

The genetic mechanisms of virulence and drug resistance are achieved through the transfer of genes and movable elements between species and genera, and virulence and drug resistance are also interrelated (3). Several studies have confirmed the relationship between drug resistance and bacteria virulence. *Streptococcus agalactiae* isolated from hospitals carried *fbsB*, *cfb*, *hylB*, *lmb*, *cylE*, *cpsA, bca*, *scpB*, *and fbsA* virulence genes aggregated in antibiotic sensitivity-related gene–rich fragments (48). This study showed that MDR strains of *S. agalactiae* ubitquitously had *hylB*, *iagA,* and *fbsA* virulence genes and had *hylB*, *iagA,* and *fbsA* in 80% of cases. It also noted that 60% of *glnA*-positive strains were resistant to TE+ERY, and 60% of the resistance genes were *ermA*+*ermB*+*lnuA*+*tetK*. The TE and ERY resistance may be due to a virulence gene. This association was supported by our analysis of antibiotic-resistance and virulence genes, but this was not statistically significant according to the binary logistic-regression model. In Shenzhen, China, 89.5–100% of *S. agalactiae* isolates from humans were resistant to tetracycline, and these isolates revealed their drug resistance gene spectrum to be *tetO*+*tetM* and their main virulence gene spectrum to be *hylB*+*lmb*+*scpB* (44). The high resistance to macrolides, lincosamides and quinolones of GBS isolates was revealed in pregnant women in southern China, and all GBS isolates harboured the *hylB* and *cylE* genes as a common virulence gene profile (15). In China, the antimicrobial-resistance and virulence gene spectra of human *S. agalactiae* evidenced by previous research are inconsistent with our results, and the relationship between the antimicrobial resistance and virulence of *S. agalactiae* is not clear and requires further exploration. However, there are serious problems with antimicrobial resistance in China, which strongly calls for the country to strengthen the monitoring of drug resistance.

In conclusion, our study showed a high prevalence of multidrug-resistant *S. agalactiae* isolated from cattle with mastitis in China. These streptococci exhibited high resistance to oxacillin, tetracycline, and erythromycin. The dominant resistance and virulence genes were *ermB* and *fbsA*, respectively. Multiple drug resistance was frequently observed in *S. agalactiae* strains expressing *iagA* and *fbsA*. However, no statistically significant correlation was observed between drug-resistance and virulence gene spectra. This study highlights the need for antimicrobial management in clinical veterinary medicine to avoid the increase and dissemination of antimicrobial resistance arising from the use of antimicrobial drugs in animals.

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