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Distribution of Serotypes, Antimicrobial Resistance and Virulence Genes among *Streptococcus agalactiae* Isolated from Bovine in China

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ABSTRACT

***Background*:** Bovine mastitis, a global disease that is responsible for large economic losses each year due to lower milk yield and reduced milk quality. In some countries, especially in China, *Streptococcus agalactiae* has become one of the most frequently detected pathogen. Antibiotic treatment and vaccine immunization are important strategies for the control of infectious diseases. The main objective of the present study was to evaluate distribution of bovine mastitis pathogens and antimicrobial resistance of *S. agalactiae*, and contribute to the treatment of bovine mastitis.

***Materials, Methods & Results*:** Clinical mastitis samples (n= 1,122) were collected from 27 dairy farms located in 15 different provinces of China during 2012-2018. The pathogens were identified by 16S rDNA method. Antimicrobial susceptibility was assessed by disc diffusion method. Molecular characteristics was distinguished based on PCR. The results showed that the main pathogens were *Streptococcus agalactiae* (n= 324, 26.2%), *Escherichia coli* (n= 287, 23.2%), and *Staphylococcus aureus* (n= 131, 10.6%). The serotypes of *Streptococcus agalactiae* were serotype II (53.6%), Ia (44 %) and VII (1.2%), respectively. *Streptococcus agalactiae* were resistant to kanamycin (93.8%), gentamicin (49.4%), vancomycin (49.4%), tetracycline (35.8%), clindamycin (34.6%) and erythromycin (32.1%). The main resistance genes were *ermA* (53.1%) and *ermB* (85.2%). Resistance to erythromycin was attributed to the genes ermA ($P < 0.05$) and resistance to tetracycline was attributed to the genes *tetK*, *tetM*, *tetO* ($P < 0.01$). The virulence genes *scpB* (81.4%), *cyl* (100%), *glnA* (76.6%), *cfb* (98.8%), *hylB* (98.8%), *scaA* (69.1%) were detected in almost all isolates.

***Discussion*:** In the present study, *Streptococcus agalactiae*, *Escherichia coli* and *Staphylococcus aureus* were the pathogens isolated most frequently from clinical mastitis. In the case of *S. agalactiae*, we performed capsular serotyping of isolates. As a result, serotype II (53.6%), Ia (44 %) and VII (1.2%) were detected which revealed variation in the distinct geographical areas. We found that serotypes (Ia and II) and β-hemolytic have significant correlation ($P < 0.01$) in all isolated strains. We made an assumption that either in processes of capsular and haemolytic appearance effected the expression of another. The unclear mechanism remains to be resolved in the future. Penicillin was recommended as a preferred antibiotic for the treatment of both human and bovine *S. agalactiae* infection. In the present study, resistance to erythromycin and clindamycin were observed in 32% and 34.6% of our strains, respectively. The results indicated that the *ermB* gene was most frequent among the erythromycin-resistant *S. agalactiae*. However, we found that the susceptibility to erythromycin and gene *ermA* have a significant interaction, while susceptibility to erythromycin and gene *ermB* have a not significant interaction by analyzing the relationship of phenotypic and genotypic resistance. The severity of *S. agalactiae* infections may be determined by various virulence factors. Surface enzyme ScpB, a C5a peptidase, encode by *scpB* gene, could promote bacterial invasion of epithelial cells by attenuating recruitment of polymorphonuclear leukocytes to the site of infection. In the present study, the *scpB* gene was found in 81.4% of all strains. The results suggested the *cyl*, *cfb*, *hylB* and *scpB* genes may play an important role in the virulence of *Streptococcus agalactiae* pathogens.

Keywords: *Streptococcus agalactiae*, serotypes, antimicrobial resistance, virulence genes.

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INTRODUCTION

Bovine mastitis, a global disease that is responsible for large economic losses each year due to lower milk yield and reduced milk quality [6,15,17,32]. Mastitis is complex, developing as a result of interaction between various factors associated with the host, specific pathogens, environment, and management [1,36]. Over 150 different bacteria have been recorded to cause bovine mastitis [5,20,33]. In China, *Streptococcus agalactiae* has become one of the most frequently detected pathogens [27,35,36].

Bacterial genotyping is one of the key steps in the development of an effective and practical vaccine. Capsular serotyping is a classical method used for *S. agalactiae* [4,18]. It is common knowledge that antibiotics are among the major tools in combating bovine mastitis. However, due to the widespread use of antimicrobial compounds, the occurrence of antimicrobial-resistant has increased [7,25]. Resistance genes are responsible for resistance to erythromycin and tetracycline which included *ermA*, *ermB*, *ermC*, *mefA*, *tetK*, *tetL*, *tetM*, *tetO* and *tetS*, respectively [7,9,11]. The ability of infections may be determined by various virulence genes, such as *cyl*, *cfb*, *hylB*, *scpB*, *lmb*, *bac*, and *scaA*, which encodes for β-hemolysin, Christie-Atkins-Munch-Peterson (CAMP), hyaluronidase, surface enzyme ScpB (C5a peptidase), laminine-binding protein, β-antigen, aggregation factor, respectively [3,19].

The aim of the present study was to evaluate distribution of bovine mastitis pathogens, antimicrobial resistance and virulence genes of *Streptococcus agalactiae* isolated from bovine mastitis cases in China, and contribute to the treatment of bovine mastitis.

MATERIALS AND METHODS

Sample collection

A total of 1,122 milk samples were collected from 27 commercial dairy farms located in 15 different provinces of China from 2012 to 2018. All samples were collected from cows with clinical mastitis aseptically as described by Pitkälä *et al*. [29]. Before sampling, first stream of milk were discarded, and the teat ends were disinfected with cotton swabs soaked in 70% (v/v) alcohol and allowed to dry. Then 5-mL secretion was collected into a sterile 10-mL tube. All samples were kept at 4°C for microbiological examination within 18 h.

Bacterial isolation and identification

The samples were enriched in 2 mL nutrient broth supplemented with 1% glucose (w/v) and 2% calf serum, and incubated at 37°C for 18-24 h. A bacteriological loop was used to spread approximately 0.02 mL of each enriched bacteria samples on blood agar[1]. The plates were incubated at 37°C and examined after 24 h. Typical *Streptococcus* colonies were distinguished microscopically by colony morphology, then by the characteristic appearance on hemolysis, and Gram's stain, catalase test and CAMP test, respectively.

The genomic DNA of strains was extracted from overnight cultured isolates using the Bacterial DNA Kit[2]. The purified genomic DNA was stored at -20°C until use. The 16S rDNA sequence was amplified by Takara 16S rDNA Bacterial Identification PCR Kit[3]. The PCR products were analyzed by electrophoresis using 1% agarose gel. By comparing the Biotechnology Information (NCBI), bacterial species were identified.

Hemolytic characteristic and capsular serotypes

Strains of *Streptococcus agalactiae* (n= 81) were randomly selected for serotyping, antimicrobial susceptibility test and antimicrobial resistance and virulence genes detection. For hemolytic characteristics of *S. agalactiae* strains, isolates were cultured on blood agar plates. Identification of serotypes was performed by multiplex PCR assay as described previously [18].

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was performed by disc diffusion method on Muller–Hinton agar plates supplemented with 5% defibrinated sheep blood[4] according to the Clinical Laboratory Standards Institute (CLSI). The antimicrobial drug discs included penicillin G (β-lactam), gentamicin (aminoglycoside), tetracycline (tetracycline), erythromycin (macrolide), kanamycin (aminoglycoside) clindamycin (lincosamide), vancomycin (glycopeptide), chloramphenicol (amphenicol), ofloxacin (fluoroquinolone) and ciprofloxacin (fluoroquinolone). After incubation at 37°C for 18-24 h, the strains could be determined as sensitive, intermediate (reduced susceptibility) or resistant characteristic by measuring the zone of inhibition according to the CLSI guidelines. We also determined the antimicrobial susceptibility of β-hemolytic and non-β-hemolytic *Streptococcus agalactiae* strains according to Clinical and Laboratory Standards Institute (CLSI 2014).

Detection of antimicrobial resistance genes and virulence genes

The antimicrobial resistance and virulence genes of strains were detected by conventional PCR as described previously [2,7,16]. The primers used for PCR amplification and sequence were shown in supplementary material.

Statistical analysis

SPSS 21.0 was used to analyze the associations between phenotypic and genotypic resistance patterns. The intermediate isolates were considered to be resistant.

RESULTS

Species identification

Out of 1,122 clinical mastitis milk samples collected and processed, 87.4% (n= 981) were culture positive for bacteria. A total of 1,235 bacterial isolates were obtained and the distribution of bacterial species was shown in Table 1.

Table 1. The distribution of bacterial and fungal species.

Pathogen	Number	Proportion (%)
Streptococcus agalactiae	324	26.2
Escherichia coli	287	23.2
Staphylococcus aureus	131	10.6
Staphylococcus epidermidis	95	7.7
Streptococcus lactis	80	6.5
Enterococcus faecalis	78	6.3
Streptococcus uberis	52	4.2
Enterococcus faecium	47	3.8
Streptococcus dysgalactiae	19	1.5
Klebsiella sp.	18	1.5
Lactococcus lactis subsp.	15	1.2
Pseudomonas aeruginosa	7	0.6
Moulds	7	0.6
Streptococcus parauberis	6	0.5
Shigella	6	0.5
Lactococcus garvieae	5	0.4
Yeasts	5	0.4
Citrobacter	5	0.4
Streptococcus pyogenes	4	0.3
Acinetobacter sp.	3	0.2
Arcanobacterium pyogenes	2	0.2
Proteus sp.	2	0.2
Undetected	37	3.0
Total	1235	100

Hemolytic characteristic and capsular serotypes

From the 81 isolates analyzed, only three serotypes were identified: Ia (44 %), II (53.6%) and VII (1.2%) as shown in Table 2. In addition, we found that the serotypes (Ia and II) and β-hemolytic have a significant correlation ($P < 0.01$) by Chi-square analysis.

Table 2. The result of serotypes and hemolysis of *Streptococcus agalactiae.*

Serotype	The type of hemolytic		Total
	β-hemolytic	Non-β-hemolytic	
Ia	40	1	41
II	2	37	39
VII	1	0	1
Total	43	38	81

Antimicrobial susceptibility

The antimicrobial resistances of *Streptococcus agalactiae* cultured from bovine mastitis were shown in Table 3. Most of them were resistant to kanamycin (93.8%). In addition, we found that 96.3% of strains were resistant to at least one of the antibiotics tested.

Detection of antimicrobial resistance genes

The presence of antibiotic resistance genes was studied by PCR in all *Streptococcus agalactiae* strains as shown in Table 4. The relationship between the antimicrobial resistance and corresponding genes were also analysed in this study. As show in Table 5, we found the susceptibility to erythromycin and gene *ermA* have a significant interaction ($P < 0.05$), the susceptibility to tetracycline and *tetK*, *tetM*, *tetO* have a strongly significant interaction ($P < 0.01$).

Detection of virulence-related genes

The presence of nine virulence-related genes was tested by PCR. The gene *cyl* was present in all *Streptococcus agalactiae* isolates. The incidence of *cfb*, *hylB*, *scpB*, *glnA*, *scaA* were 98.8%, 98.8%, 81.4%, 76.6% and 69.1%, respectively.

DISCUSSION

Isolation and identification of mastitis pathogens

Pathogens as a cause of mastitis have become the major concern to the dairy industry worldwide due to huge economic losses. In the present study, *Streptococcus agalactiae*, *Escherichia coli* and *Staphylococcus aureus*

Table 3. Antimicrobial susceptibility of *Streptococcus agalactiae*.

Antimicrobial agent	Antimicrobial susceptibility					
	R*		I*		S*	
	n	%	n	%	n	%
Penicillin	0	0	0	0	81	100
Kanamycin	76	93.8	4	4.9	1	1.2
Gentamicin	40	49.4	29	35.8	12	14.8
Ofloxacin	4	4.9	10	12.3	67	82.7
Ciprofloxacin	9	11.1	27	33.3	45	55.6
Chloramphenicol	4	4.9	15	18.5	62	76.5
Erythromycin	26	32.1	2	2.5	53	65.4
Tetracycline	29	35.8	10	12.3	42	51.9
Clindamycin	28	34.6	0	0	53	65.4
Vancomycin	40	49.4	0	0	41	50.6

* R: resistant; I: intermediate; S: sensitive. The same as below.

Table 4. Distribution of antibiotic-resistance genes among *Streptococcus agalactiae.*

Target gene	The number of strains with target gene	percentage
ermA	43	53.1%
ermB	69	85.2%
ermC	3	3.7%
mefA	0	0%
tetK	32	39.5%
tetL	0	0%
tetM	24	29.6%
tetO	24	29.6%
tetS	32	39.5%

Table 5. Comparison of phenotypic and genotypic test for antimicrobial resistance.

Antimicrobials	Gene(s)	Characteristics of *Streptococcus agalactiae**					*P* value
		P+/G+ (n)	P–/G–(n)	P+/G–(n)	P–/G+ (n)	G+(%)	
Erythromycin	ermA	20	22	23	6	79.2%	0.032
	ermB	24	10	45	2	94.3%	0.214
	ermC	1	53	2	25	1.9%	0.963
	mefA	0	26	0	55	0	--
Tetracycline	tetK	20	41	12	9	69.0%	<0.001
	tetL	0	53	0	29	0	--
	tetM	22	51	2	7	75.9%	<0.001
	tetO	18	47	6	11	62.1%	<0.001
	tetS	12	33	20	27	41.4%	0.797

*P+: phenotypic resistance, P–: phenotypic susceptibility, G+: resistant gene positive, G–: resistant gene negative.

were the pathogens isolated most frequently from clinical mastitis. The same results were shown in previous reports [20,33,35]. Zhang *et al*. [36] observed that *S. agalactiae* was the bacterium isolated most frequently (38.6%) from milk samples of mastitis cases in China, the result was similar with our date (26.2%). In some other countries, the isolation rate of *S. agalactiae* has been reported to be 60% in Brazil and 42% in Colombia [8,23], while lower isolation rate was found in Danish (7%) and Ragusa (2%) [14,21]. In this study, the proportions of *E. coli* and *S. aureus* were 23.3% and 10.6%, respectively. The same results were shown in an Estonia study, where *E. coli* (15.9%) and *S. aureus* (11.7%) were the pathogens isolated most commonly from case of clinical mastitis [21,26]. However, the results were different to previous reports where *E. coli* (7%) and *S. aureus* (74%) [20]. The prevalence of dominant mastitis pathogens differs considerably among countries. Even in the same country, it is always different among regions due to different management level and climate.

Capsular serotypes and haemolytic characteristic

In the case of *Streptococcus agalactiae*, we performed additional molecular serotyping of the isolated strains. Such investigations are extremely important considering both epidemiology and prophylaxis, as they may aid the development of multivalent vaccines containing capsular polysaccharides [35]. Several methods have been used to investigate the serotyping of *S. agalactiae*. Among them, capsular serotyping is a classical method used for *S. agalactiae* in epidemiological studies. To date, ten serotypes, based on the *S. agalactiae* capsular polysaccharides (cps), have been identified, including Ia, Ib, II-VIII and a new serotype IX [4,18]. In the present study, serotype II (53.6%), Ia (44 %) and VII (1.2%) were detected from *S. agalactiae* strains isolated from clinical cows with mastitis [35], our finding are coincident with the previously reported data, but the prevalence of the each serotype was variable and we also identified one serotype VII. Indeed, the data of serotype distribution of *S. agalactiae* associated with bovine mastitis have revealed considerable variation in the distinct geographical areas. Serotype Ia, □, □, IV and V reported previously were the most prevalent in the Brazil, Canada and Norway, respectively [28,30,37].

In the present study, we also identified haemolytic characteristic of all *S. agalactiae* strains. We concluded that serotypes (Ia and II) and β-hemolytic have significant correlation ($P < 0.01$) in all isolated strains. We made an assumption that either in processes of capsular and haemolytic appearance effected the expression of another. The unclear mechanism remains to be resolved in the future.

Phenotypic and genotypic resistance

The present study showed that 81 *Streptococcus agalactiae* isolates tested against 10 antibiotics demonstrated the existence of a variable and broad antimicrobial resistance profile among local *S. agalactiae*. Penicillin was recommended as a preferred antibiotic for the treatment of both human and bovine *S. agalactiae* infection. Our data showed that all strains were susceptibility to penicillin which was in agreement with the previously reported data about the high levels of susceptibility to penicillin of mastitis *S. agalactiae* [12,24,31]. But one study from Inner Mongolia, China, reported penicillin resistant *S. agalactiae*, despite of decreased susceptibility to penicillin, it is still a preferred antibiotic used for treatment of *S. agalactiae* mastitis in China [7].

Erythromycin and clindamycin are recommended for penicillin-allergic individuals. However, because of extensive use of erythromycin and clindamycin, the rate of strains which were resistant to erythromycin have been described in Brazil from 10.5% between 1987 and 1988 to 60% between 2003 and 2006 [28]. In the present study, resistance to erythromycin and clindamycin were observed in 32% and 34.6% of our strains, respectively. This result is lower than those reported in Inner Mongolia, China [7].

Erythromycin and clindamycin belong to antimicrobial categories of macrolides and lincosamides, respectively. The products of erm genes confer combined resistance to macrolides and lincosamides by methylation of the ribosomal binding site of these drugs [4,11] and the mef gene is another major resistant factor of resistance to erythromycin. We detected the related genes *ermA* (53.1%), *ermB* (85.2%), *ermC* (3.7%) in all strains, while the strains of resistance to erythromycin harbored genes *ermA* (79.2%), *ermB* (94.3%), *ermC* (1.9%), respectively. None of the tested strains were positive for *mefA*. The results indicated that the *ermB* gene was most frequent among the erythromycin-resistant *S. agalactiae*, which is in accordance with other studies [11,28]. However, we found that the susceptibility to erythromycin and gene *ermA* have a significant interaction, while susceptibility

to erythromycin and gene *ermB* have a not significant interaction by analyzing the relationship of phenotypic and genotypic resistance.

Tetracycline is one of the main antimicrobials in the prevention and control of bovine mastitis [22,34]. Because of the vast and irrational use of tetracycline, the rate of tetracycline-resistant strains has been increased. Some reports have showed that the rate of tetracycline-resistant *S. agalactiae* from cattle was considerably higher than previous data in Brazil and in the United States [28]. Our result showed that the rate of tetracycline-resistant strains was 35.8%, which was much lower than described (61.7%) previously in Inner Mongolia, China [7]. Resistance to tetracycline is usually mediated either by active efflux of tetracycline from the cell (*tetL* and *tetK*) or by ribosomal protection (*tetM*, *tetO*, *tetS*, and *tetT*) from the action of tetracycline. In this present study, the genes *tetK*, *tetM*, *tetO* and *tetS* were harbored in 39.5%, 29.6%, 29.6% and 39.5% of all strains respectively, while no gene *tetL* was detected. The tetracycline-resistant strains carried at least one tet gene. The result was in accordance with previous report [7]. After analyzing the susceptibility to tetracycline and genes tet, the result indicated resistance to tetracycline was attributed to the genes *tetK*, *tetM* or *tetO* ($P < 0.01$) which are in according to previous study of *Staphylococcus aureus* [35].

In this study, all strains had a high frequency of phenotypic resistance to kanamycin (98.8%) and gentamicin (85.2%), which belonged to aminoglycosides. The rate of non-sensitive isolates from the mastitis milk of dairy cows were 54.8% (kanamycin) and 16.1% (gentamicin) in Shahrekord district, Iran [9]. In another study the rate of gentamicin-non-sensitive *Streptococcus agalactiae* isolates from bovine mastitis cases was 76.9% in Michigan [12]. In the contrast, the percentage of the gentamicin-resistance isolates from cases of bovine subclinical mastitis was found in 3.7% in India [19].

In addition, phenotypic resistance to partial pairs antibiotic agents have significant interaction. Excepting the antibiotic agents belonged to the same categories or antibiotic agents had the same resistant mechanisms, the mechanisms need to further study in the pairs antibiotic agents with significant interaction.

Virulence genes

The severity of *Streptococcus agalactiae* infections may be determined by various virulence factors. Surface enzyme ScpB, a C5a peptidase, encode by *scpB* gene, could promote bacterial invasion of epithelial cells by attenuating recruitment of polymorphonuclear leukocytes to the site of infection [3,19]. In the present study, the *scpB* gene was found in 81.4% of all strains. The result was similar to the previous reported strains from human in Kuwait [10], while lower prevalence data was found in bovine strains in India and in Inner Mongolia, China [16,35]. Some reports showed the gene *scpB* was found more frequent in human than in bovine [10]. However, our result was not in accordance with these findings. The gene *cfb* encodes forceramide-binding protein. According to the studies of Shome *et al*. [33], all *S. agalactiae* isolates carried the *cfb* gene, the result was similar with our date. The genes *cyl* and *hylB* encode for b-hemolysin and group B *Streptococcal hyaluronate* lyase, respectively. In the present study, 100% isolates had the *cyl* gene, 98.8% isolates had the *hylB* gene. Ding *et al*. [7] reported that *cyl* and *hylB* were founded to be 48.1% and 49.4%, while Eskandarian *et al*. [13] reported that *cylE* and *hylB* were found in 97.1% and 94.2% in human strains, respectively. The gene *lmb*, *bac*, and *bca* code laminin binding protein, β-C protein, α-C protein respectively. Our result showed 24.7%, 12.3%, 49.3% strains of all harbored *lmb*, *bac*, and *bca*, respectively. These observations are in agreement with earlier reports [7]. The results suggested the *cyl*, *cfb*, *hylB* and *scpB* genes may play an important role in the virulence of *S. agalactiae* pathogens.

CONCLUSIONS

This study showed that a relatively high number of isolates of *Streptococcus agalactiae* was cultured from milk samples of bovine clinical mastitis in China. The serotype a and serotype were the predominant serotypes in the *S. agalactiae* strains isolated from cases of bovine mastitis. The serotype distribution of the *S. agalactiae* strains could provide the basis for developing the bovine mastitis vaccine. Besides, our study demonstrated that *S. agalactiae* from bovine mastitis in China are resistant to many of the antimicrobial compounds commonly used for treatment of mastitis. Resistance to erythromycin was attributed to the genes *ermA* ($P < 0.05$) and resistance to tetracycline was attributed to the genes *tetK*, *tetM*, *tetO* ($P < 0.01$). In addition, the results indicated that the virulence genes *cyl*, *cfb*, *hylB* were preponderant virulence genes in *S. agalactiae*.

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