Phenotypic and genotypic characterization of antimicrobial resistance profiles in *Streptococcus dysgalactiae* isolated from bovine clinical mastitis in 5 provinces of China

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ABSTRACT

Bovine mastitis is among the most prevalent and costly diseases of dairy animals and is caused by a variety of bacterial pathogens including *Streptococcus dysgalactiae*. However, comprehensive studies reporting the prevalence and antimicrobial resistance profiles of *S. dysgalactiae* isolated from bovine mastitis are scarce. Therefore, this study was to investigate the occurrence of *S. dysgalactiae* associated with bovine clinical mastitis, to assess their antimicrobial resistance profiles, and to analyze the phenotypic and genotypic profiling of resistant isolates. In total, 1,180 milk samples were collected from dairy cows with clinical mastitis belonging to 74 commercial dairy herds located in 14 provinces of China from January 2014 to May 2016. Overall *S. dysgalactiae* isolates were recovered from 88 (7.5%) of the mastitic milk samples. The antimicrobial susceptibility of these isolates was tested against 8 antimicrobial agents by using minimum inhibitory concentrations. Results showed that 82 (93.2%) isolates expressed resistance to more than one antimicrobial agent. Antimicrobial resistance was highest against kanamycin (89.8%), sulfonamide (83.0%), and streptomycin (58.0%), which can be attributed to the intrinsic resistance for most of *Streptococcus* spp. against those antimicrobial substances. Strikingly, 30 (34.1%) and 12 (13.6%) isolates were found resistant to cephalexin and ceftriaxone, respectively. *BlaTEM*, *ermB*, and *tetM* were the most prevalent resistance genes. All isolates carried at least one of all tested resistance genes. Also, 1.1, 12.5, 18.2, 36.4, and 31.8% of isolates were positive for at least one tested resistance gene in 1, 2, 3, 4, or 5 classes of antimicrobials. Survival analysis showed a

significant association between *ermB* and survival of the *S. dysgalactiae* isolates at increasing erythromycin concentrations. No other statistically significant associations were observed between the phenotypic and genotypic resistance profiles. This study concludes a considerable prevalence of *S. dysgalactiae* associated with bovine mastitis in dairy herds of China and these isolates exhibited high resistance rates to tested antimicrobials, coupled with high occurrence of resistance genes. Both the prevalence of *S. dysgalactiae* and their antimicrobial resistance profiles strongly varied among dairy herds, demonstrating the need for antimicrobial susceptibility surveillance at the herd level to ensure optimal therapeutic results.

Key words: *Streptococcus dysgalactiae*, antimicrobial resistance, bovine clinical mastitis

INTRODUCTION

Bovine mastitis, inflammation of the mammary gland, is still the most prevalent disease in dairy cattle. The inflammatory reaction primarily occurs in response to bacterial IMI and impairs milk quality (Petrovski et al., 2006; Halasa et al., 2007). This disease also accounts for the highest proportion of antimicrobial usage on a dairy farm (Saini et al., 2012; Kuipers et al., 2016; Stevens et al., 2016). It is estimated that 60 to 70% of all antimicrobials used on dairy farms is applied for the prevention and control of mastitis, of which roughly half is related to the treatment of clinical mastitis (Kuipers et al., 2016; Stevens et al., 2016). Mastitis is characterized by clinical signs of inflammation and alterations in the appearance of milk (i.e., clinical mastitis) or it may be without any visual abnormalities (i.e., subclinical mastitis). Clinical and subclinical mastitis also affect animal welfare (Peters et al., 2015).

Apart from one recent research work in which the prevalence of the most common mastitis pathogens was

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studied in bulk milk samples collected from 894 Chinese dairy farms (Bi et al., 2016) and one recent report about the incidence of clinical mastitis and distribution of pathogens on 161 large Chinese dairy farms (Gao et al., 2017), no other information is available on the prevalence and distribution of mastitis pathogens in Chinese dairy farms. Of particular interest in the first study was the high prevalence of *Streptococcus dysgalactiae*-positive bulk milk samples; *S. dysgalactiae* was detected in 72.3% of the bulk milk samples (Bi et al., 2016). It was noteworthy that the prevalence of *S. dysgalactiae* was significantly higher than the prevalence of other major mastitis pathogens such as *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Streptococcus uberis* (Gao et al., 2017), as well as in most other countries including Norway, Belgium, France, and Thailand (Piepers et al., 2007; Whist et al., 2007; Botrel et al., 2010; Leelahapongsathon et al., 2014). Still, bacteria in bulk milk do not solely originate from infected quarters. Environmental contamination or insufficient hygiene and poor preservation conditions can also cause positive bulk milk samples (Elmoslemany et al., 2009a,b). To get better insights in the exact relevance of *S. dysgalactiae* for the udder health in dairy cows in China, studies on the prevalence of *S. dysgalactiae* in clinical mastitis are required.

Despite the increasing pressure to reduce antimicrobial use in food-producing animals, antimicrobials still have important benefits for animal health and are still an essential tool in mastitis control programs (Middleton et al., 2014). Failure or success of treatment of clinical mastitis depends on several factors including those related to the cow such as age or parity, stage of lactation (DIM), SCC, and clinical mastitis history, and those related to the pathogen such as virulence and antimicrobial susceptibility (Sol et al., 2000; Taponen et al., 2003; Barkema et al., 2006). Antimicrobial resistance surveillance studies are imperative to choose the most appropriate antimicrobial therapies and to reduce the risk for further development and spread of antimicrobial resistance through lateral transfer of resistance genes or direct transfer of resistant pathogens. Some information is available on the prevalence of phenotypic antimicrobial resistance in *S. dysgalactiae* (Guérin-Faubleé et al., 2002; Bengtsson et al., 2009; McDougall et al., 2014; Petrovski et al., 2015), though no studies have comprehensively investigated the genotypic antimicrobial resistance in *S. dysgalactiae*.

The main objective of the current study was to estimate the prevalence of clinical mastitis caused by *S. dysgalactiae* in Chinese dairy herds and to determine the prevalence of both phenotypic and genotypic antimicrobial resistance profiles of the collected *S. dysgalactiae* isolates. Additionally, results obtained by phenotypic

and genotypic profiling of antimicrobial resistance were compared.

MATERIALS AND METHODS

Data and Sample Collection

Streptococcus dysgalactiae isolates included in this study were collected from milk samples of dairy cows with clinical mastitis ($n = 1,180$). In total, 74 dairy farms located in 14 different provinces were involved in the study (Beijing = 23 milk samples; Tianjin = 85 samples; Hebei = 196 samples; Shandong = 87 samples; Shaanxi = 26 samples; Shanxi = 48 samples; Inner Mongolia = 321 samples; Guangdong = 98 samples; Shanghai = 47 samples; Henan = 17 samples; Anhui = 78 samples; Heilongjiang = 56 samples; Jilin = 25 samples; Liaoning = 73 samples). On each farm, all the dairy farms veterinarians were requested to report the signs of clinical mastitis cases along with aseptically collected milk samples between January 2014 and May 2016. In case that only one quarter was affected, sample was taken from the quarter showing signs of clinical mastitis. If 2 or more quarters showed clinical signs at the same time, a composite milk sample from all quarters showing clinical signs [any abnormal aspect of the milk (flakes, clots, and a watery or other unusual appearance) with or without other visible abnormalities of the udder (e.g., redness, swelling, firmness)] was collected. Sample materials were provided and sampling procedures were explained in light of National Mastitis Council (NMC, 1999) instructions as well as the importance of an aseptic sampling procedure. Samples taken from the same cow within 3 mo from a previous case were excluded from the analysis. All the milk samples for bacteriological culturing were transported in ice boxes to our Bovine Mastitis Research Laboratory at the College of Veterinary Medicine, China Agricultural University, Beijing, China.

Microbiology

A volume of 30 μ L from each of the composite milk samples was plated on a tryptic soy agar (TSA; Oxoid, Basingstoke, UK) containing 5% defibrinated sheep blood (Land Bridge Technology, Beijing, China) and incubated aerobically at 37°C for 24 h. Subsequently, the identification of *Streptococcus dysgalactiae* was carried out by Gram staining, colony morphology, and biochemical tests. Streptococci (catalase-negative) were differentiated from staphylococci (catalase-positive) using catalase test. Isolates of the *Streptococcus-Enterococcus* group were differentiated as esculin-positive (*Streptococcus uberis* and other esculin-positive cocci)

or esculin-negative cocci (*S. dysgalactiae* and *S. agalactiae*). Christie, Atkins, and Munch-Petersen (CAMP) test was used to differentiate *S. dysgalactiae* (CAMP-negative) from *Streptococcus agalactiae* (CAMP-positive). Samples yielding 2 different bacterial colonies were categorized as mixed culture. However, those samples which yielded 3 or more different bacterial colonies were considered contaminated.

In case that a sample was positive for *S. dysgalactiae* in pure or mixed culture, only one *S. dysgalactiae* colony was picked up per plate (i.e., per sample) for further analysis. All recovered isolates suspected of being *S. dysgalactiae* (n = 88) were subjected to 16S rRNA gene sequencing for the *S. dysgalactiae* species identification using universal primers as described previously (Frank et al., 2008). The purified PCR products (750 bp) were sequenced by an automated ABI 3730 sequencer (Beijing Sunbiotech Inc., Beijing, China). The sequences were compared using Basic Local Alignment Search Tool tools at the National Center for Biotechnology Information, and species were identified at 98% or above homology. All the isolates (n = 88) were confirmed as *S. dysgalactiae*, which were then stored in Brain Heart infusion broth (Aobox Biotechnology, Beijing, China) containing 25% glycerol at -80°C until further processing.

Phenotypic Resistance Profiling

The stored isolates were thawed at 37°C . First, the isolates were grown on TSA blood agar plates (Oxoid). Then, fresh colonies were grown in Mueller-Hinton broth (Oxoid) containing 5% fetal bovine serum (Tianhang Biotechnology, Hangzhou, China) for 18 h at 37°C . After bacterial growth, the bacterial suspension was plated on TSA blood agar plates in a serial dilution from 10^{-1} to 10^{-12} and the colonies were counted after 20 h. The inoculum was adjusted to 5×10^5 cfu/mL.

Antimicrobial susceptibility of all the clinical *S. dysgalactiae* isolates was tested against a panel of antimicrobials that are routinely used on Chinese dairy farms, by using standard microdilution broth method. The antimicrobial agents included cephalixin (≥ 4 $\mu\text{g/mL}$), ceftriaxone (≥ 4 $\mu\text{g/mL}$), erythromycin (≥ 1 $\mu\text{g/mL}$), kanamycin (≥ 16 $\mu\text{g/mL}$), norfloxacin (≥ 8 $\mu\text{g/mL}$), streptomycin (≥ 32 $\mu\text{g/mL}$), sulfonamide (≥ 32 $\mu\text{g/mL}$), and tetracycline (≥ 8 $\mu\text{g/mL}$) using the guidelines and breakpoints of Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2015) where available. For this purpose, cationic-adjusted Mueller-Hinton broth (Oxoid) was supplemented with 5% lysed horse blood. Because no specific resistance breakpoints for *Streptococcus* spp. were available for some tested antimicrobials, the resistance break-

points for either an antimicrobial of the same antimicrobial drug class (for cephalixin and norfloxacin) or another pathogen group [i.e., *Enterobacteriaceae* (for sulfonamide and kanamycin)] were referred to as recommended by the Clinical and Laboratory Standards Institute (2015). Minimum inhibitory concentrations were determined initially using a reference strain of *Streptococcus pneumoniae* ATCC 49619.

Antimicrobial Resistance Genotyping

Genotypic resistance profiles of clinical *S. dysgalactiae* isolates were determined by PCR, assays targeting the genes encoding resistance to aminoglycosides, β -lactams, macrolide, quinolones, streptomycin, sulfonamides, and tetracyclines. Primers and PCR conditions were as previously described (Table 1).

All primers were commercially synthesized (Sunbiotech Inc.). To extract DNA, bacterial cells were suspended in double-distilled water (TransGen Biotech, Beijing, China) and boiled for 20 min. Subsequently, the cells were pelleted by centrifugation ($504 \times g$, at room temperature) for 1 min. Amplifications were carried out with 5 μL of the supernatants as DNA template (Matayoshi et al., 2015). The PCR was performed in a final volume of 25 μL using $2 \times$ EasyTaq PCR SuperMix (+ dye; TransGen Biotech, Beijing, China). The PCR was performed with the following parameters: an initial denaturation cycle at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 58°C (52 – 66°C , determined by the primer) for 30 s, elongation at 72°C for 50 s, and a final cycle at 72°C for 10 min.

All PCR products were submitted to Sunbiotech Inc. for sequencing. The resulting DNA sequence data were compared with data in the GenBank database using the Basic Local Alignment Search Tool algorithm available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

Statistical Analysis

The frequency distributions of the MIC values for each antimicrobial were tabulated and presented graphically. The antimicrobial concentration at which 50 and 90% of the isolates were inhibited were represented by MIC_{50} and MIC_{90} values, respectively.

Survival analysis was used to plot survival of *S. dysgalactiae* isolates with and without specified resistance genes against MIC values from relevant phenotypic assays. In these analysis, the time-to-event was given by the MIC. When an organism was still growing at the specified antimicrobial concentration, the observation was coded as censored. The Kaplan-Meier estimator

Table 1. Detailed information of the primers used to detect antimicrobial resistance genes in this study

Antimicrobial drug class	Resistance gene	Primer sequence (5'→3')	PCR product size (bp)	Reference
Aminoglycosides	<i>aphA-1</i>	ATGGGCTCGCGATAATGTC CTCACCAGGAGCAGTTCCAT	600	Sáenz et al., 2004
	<i>aphA-2</i>	GAACAAGATGGATTGCACGC GCTCTTCAGCAATATCACGG	680	Sáenz et al., 2004
	<i>aphA-3</i>	GGGGTACCTTTAAATACTGTAG TCTGGATCCTAAAACAATTCATCC	848	Poyart et al., 2003
	<i>aadA1/aadA2</i>	GCAGCGCAATGACATTCTTG ATCCTTCGGCGCGATTTTG	282	Sáenz et al., 2004
	<i>aad-6</i>	AGAAGATGTAATAATATAG CTGTAATCACTGTTCCCGCCT	978	Poyart et al., 2003
	<i>blaIMP</i>	CTACCGCAGCAGAGTCTTTG AACCAGTTTTGCCTTACCAT	587	Sundsford et al., 2004
β-Lactams	<i>blaSHV</i>	ATGCGTTATATTCGCCTGTG TTAGCGTTGCCAGTGCTCGA	860	Sundsford et al., 2004
	<i>blaSPM-1</i>	CCTACAATCTAACGGCGACC TCGCCGTGTCCAGGTATAAC	649	Sundsford et al., 2004
	<i>blaTEM</i>	ATGAGTATTC AACATTTTCGTG TTACCAATGCTTAATCAGTGAG	860	Sundsford et al., 2004
	<i>blaVIM</i>	ATTCCGGTCGGAGAGGTCCG GAGCAAGTCTAGACCGCCCG	633	Sundsford et al., 2004
	<i>mecA</i>	TGGCTATCGTGT CACAATCG CTGGAAC TTGTTGAGCAGAG	310	Sundsford et al., 2004
	<i>ermA/TR</i>	TCAGGAAAAGGACATTTTACC ATACTTTTTGTAGTCTCTTCTT	432	Sutcliffe et al., 1996
Macrolides	<i>ermB</i>	GAAAAGGTACTCAACCAAATA AGTAACGGTACTTAAATTGTTTAC	639	Sutcliffe et al., 1996
	<i>ermC</i>	TCAAAACATAATATAGATAAA GCTAATATTGTTTAAATCGTCAA	642	Sutcliffe et al., 1996
	<i>mdfA/mefE</i>	AGTATCATTAATCACTAGTGC TTCTTCTGGTACTAAAAGTGG	348	Sutcliffe et al., 1996
	<i>gyrA</i>	TTCTCCGATTTCCTCATG AGAAGGGTACGAATGTGG	458	de Toro et al., 2010
Quinolones	<i>parC</i>	TGGGTTGAAGCCGGTTCA CAAGACCGTTGGTTCTTTTC	361	de Toro et al., 2010
	<i>rpsL</i>	GGCCGACAAAACAGAACGT GTTCCACCAACTGGGTGAC	501	Sreevatsan et al., 1996
Streptomycin	<i>rrs</i>	GAGAGTTTGATCCTGGCTCAG TGCACACAGGCCACAAGGGA	1,042	Sreevatsan et al., 1996
Sulfonamides	<i>sul1</i>	TGGTGACGGTGTTCGGCATTC GCGAGGGTTTCCGAGAAGGTG	789	Sáenz et al., 2004
	<i>sul2</i>	CGGCATCGTCAACATAACC GTGTGCGGATGAAGTCAG	722	Sáenz et al., 2004
	<i>sul3</i>	CATTCTAGAAAACAGTCGTAGTTTCG CATCTGCAGCTAACCTAGGGCTTTGGA	990	Sáenz et al., 2004
	<i>tetA</i>	GTAATTCTGAGCACTGTCGC CTGCCTGGACAACATTGCTT	937	Sáenz et al., 2004
Tetracyclines	<i>tetB</i>	CTCAGTATTCCAAGCCTTTG CTAAGCACTTGTCTCCTGTT	416	Sáenz et al., 2004
	<i>tetC</i>	TCTAACAATGCGCTCATCGT GGTTGAAGGCTCTCAAGGGC	570	Sáenz et al., 2004
	<i>tetD</i>	ATTACACTGCTGGACGCGAT CTGATCAGCAGACAGATTGC	1,104	Sáenz et al., 2004
	<i>tetE</i>	GTGATGATGGCACTGGTCAT CTCTGCTGTACATCGTCTT	1,179	Sáenz et al., 2004
	<i>tetK</i>	GTAGGATCTGCTGCATTCCC CACTATTACCTATTGTCGC	552	Poyart et al., 2003
	<i>tetL</i>	GGATCGATAGTAGCCATGGG GTATCCCACCAATGTAGCCG	516	Poyart et al., 2003
	<i>tetM</i>	GTGGAGTACTACATTACGAG GAAGCGGATCACTATCTGAG	359	Poyart et al., 2003
	<i>tetO</i>	GCGGAACATTGCATTTGAGGG CTCTATGGACAACCCGACAGAAG	538	Poyart et al., 2003

Figure 1. Distribution of *Streptococcus dysgalactiae* isolates recovered from milk samples of bovine clinical mastitis cases collected on Chinese dairy farms stratified by province. GD = Guangdong; HB = Hebei; IM = Inner Mongolia; SH = Shanghai; and SN = Shandong.

was used to estimate survival curves and the log-rank test was used to test for significant associations between gene presence and MIC value (Sampimon et al., 2011). The analysis was only performed for those resistance genes that were carried by at least 2 isolates. Hazard ratios and 95% confidence interval were calculated. Statistical significance was defined at $P < 0.05$.

RESULTS

Isolation and Identification of *Streptococcus dysgalactiae*

Overall, 88 clinical mastitis cases were culture positive for *Streptococcus dysgalactiae*. The prevalence of *S. dysgalactiae* was 7.5% from 1,180 milk samples collected from dairy cows with clinical mastitis belonging to 74 commercial dairy herds located in 14 different provinces of China. Eighty-eight *S. dysgalactiae* isolates were isolated from 88 cows (i.e., one isolate from one cow) belonging to 17 dairy herds located in the 5 provinces

[i.e., Guangdong (n = 3), Hebei (n = 13), Inner Mongolia (n = 68), Shanghai (n = 1), and Shandong (n = 3); Figure 1 and Table 2]. Of the 88 *S. dysgalactiae* isolates, 19 isolates were recovered in pure culture, and 69 isolates were recovered in combination with other mastitis pathogens, such as *Escherichia coli* and CNS. A high variation in the prevalence of *S. dysgalactiae* was observed among different dairy herds.

Phenotypic Resistance Profiles of *Streptococcus dysgalactiae*

Two out of 88 (2.3%) isolates were phenotypically susceptible to all the tested antimicrobials using the Clinical and Laboratory Standards Institute breakpoints. Four (4.5%) isolates expressed resistance to a single compound, and 82 isolates (93.1%) showed resistance to more than one antimicrobial agent. Thirteen isolates were expressing resistance to 2 related compounds (i.e., to kanamycin and streptomycin), whereas 69 isolates were found to be resistant to compounds belonging to

Table 2. Antimicrobial resistance rates of 88 *Streptococcus dysgalactiae* isolates recovered from milk samples of bovine clinical mastitis cases collected in 17 dairy herds stratified by province

Antimicrobial drug	No. (%) of resistant isolates stratified by province					Total % (n = 88)
	Guangdong (n = 3)	Hebei (n = 13)	Inner Mongolia (n = 68)	Shanghai (n = 1)	Shandong (n = 3)	
Cephalexin	0 (0)	0 (0)	30 (44.1)	0 (0)	0 (0)	34.1
Ceftriaxone	1 (33.3)	0 (0)	11 (16.2)	0 (0)	0 (0)	13.6
Erythromycin	0 (0)	4 (30.8)	37 (54.4)	0 (0)	1 (33.3)	47.7
Kanamycin	3 (100)	11 (84.6)	61 (89.7)	1 (100)	3 (100)	89.8
Norfloxacin	1 (33.3)	4 (30.8)	10 (14.7)	0 (0)	1 (33.3)	18.2
Streptomycin	1 (33.3)	7 (53.8)	42 (61.8)	1 (100)	1 (33.3)	58.0
Sulfonamide	3 (100)	4 (30.8)	57 (83.8)	1 (100)	3 (100)	83.0
Tetracycline	1 (33.3)	1 (7.7)	27 (39.7)	0 (0)	0 (0)	33.0

Table 3. Minimum inhibitory concentrations of *Streptococcus dysgalactiae* isolates recovered from milk samples of bovine clinical mastitis cases (n = 88) showing MIC₅₀ and MIC₉₀¹

Antimicrobial agent	Range of antimicrobial (µg/mL)	Resistance breakpoint ² (µg/mL)	MIC range (µg/mL)	MIC ₅₀ (µg/mL)	MIC ₉₀ (µg/mL)	Resistance rate (%)
Cephalexin	0.03–256	≥4	0.06–128	0.5	8	34.1
Ceftriaxone	0.03–256	≥4	0.06–64	0.06	4	13.6
Erythromycin	0.03–256	≥1	0.06–128	0.5	128	47.7
Kanamycin	0.03–256	≥16	0.5–128	64	128	89.8
Norfloxacin	0.03–256	≥8	0.25–64	2	17.6	18.2
Streptomycin	0.5–256	≥32	1–128	64	128	58.0
Sulfonamide	0.5–256	≥32	4–128	64	128	83.0
Tetracycline	0.03–256	≥8	1–128	4	70.4	33.0

¹MIC₅₀ represents the antimicrobial concentration at which 50% of the isolates were inhibited. MIC₉₀ represents the antimicrobial concentration at which 90% of the isolates were inhibited.

²Resistance breakpoints based on Clinical and Laboratory Standards Institute (2015).

different antimicrobial classes (Figure 2). Only 12 of 30 isolates that showed resistance against cephalexin were also found to be resistant to ceftriaxone. The antimicrobial agents to which antimicrobial resistance was demonstrated most frequently were kanamycin (89.8%), sulfonamide (83.0%), and streptomycin (58.0%). Ceftriaxone (13.6%) and norfloxacin (18.2%) were the antimicrobial agents with the lowest level of antimicrobial resistance. Importantly, 30 (34.1%) and 12 (13.6%) isolates were also found to be resistant against the first (cephalexin) and third (ceftriaxone) generation cephalosporins, respectively (Table 3).

Also, the lowest MIC₅₀ and MIC₉₀ values were observed for ceftriaxone (0.06 and 4 µg/mL, respectively)

and cephalexin (0.5 and 8 µg/mL, respectively), whereas the highest MIC₅₀ and MIC₉₀ values were noted for kanamycin, streptomycin, and sulfonamide (64 and 128 µg/mL, respectively; Table 3).

Herds 9, 10, 11, and 12 had a relatively higher prevalence of *S. dysgalactiae* as compared with other herds. Moreover, in those 4 herds various groups of resistant *S. dysgalactiae* isolates exhibiting different resistant patterns were observed (Table 4). For example, in herd 9, 5 categories of resistant *S. dysgalactiae* isolates could be distinguished, and 9, 12, and 9 categories in herd 10, 11, and 12, respectively. The latter categories showed resistance to 2, 3, 5, 6, and 7 different classes of antimicrobials, respectively.

Figure 2. Distribution of phenotypically resistant *Streptococcus dysgalactiae* isolates recovered from milk samples of bovine clinical mastitis cases collected on Chinese dairy herds. Different numbers of antimicrobial drugs (i.e., 0, 1, 2, 3, 4, 5, 6, or 7) showed resistance to various clinical isolates from mastitic cows in 17 dairy herds. 0 = no isolate was resistant to any tested drug; 1 = isolates resistant to one drug; 2 = isolates resistant to 2 drugs, and so on.

Table 4. Number of *Streptococcus dysgalactiae* isolates with different phenotypic resistance profiles isolated from the 4 herds with the highest prevalence of *Streptococcus dysgalactiae*

Antimicrobial drug	Herd 9					Herd 10								Herd 11											Herd 12									
	2 ¹	4	2	2	2	1	1	3	1	1	1	1	1	1	2	2	3	1	1	1	3	1	1	7	1	1	2	1	1	1	3	3		
Cephalexin	− ²	−	−	−	+ ³	+	+	+	+	+	+	−	+	+	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	+	−	−	+	+
Ceftriaxone	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	+	−	−	+	−	−	−	−	−	−	+	−
Erythromycin	−	−	−	+	+	−	−	−	−	+	+	+	+	+	−	−	−	−	+	+	+	+	+	+	+	−	+	−	−	+	+	+	+	+
Kanamycin	+	+	+	+	+	−	+	+	+	−	+	+	+	+	−	+	+	−	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+
Norfloxacin	−	−	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	
Streptomycin	+	+	+	+	−	+	−	+	+	+	+	+	+	−	−	−	+	+	−	−	+	−	+	+	+	+	−	+	−	−	+	−	−	−
Sulfonamide	−	+	+	+	+	−	+	+	−	−	+	+	+	+	−	−	−	+	−	+	−	+	−	+	+	+	+	+	+	+	+	+	+	+
Tetracyclin	−	−	+	+	+	−	−	−	−	+	−	+	+	+	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	+	+

¹Number of resistant isolates.
²− = absence of phenotypic resistance against the specific antimicrobial.
³+ = presence of phenotypic resistance against the specific antimicrobial.

Genotypic Resistance Profiles of *Streptococcus dysgalactiae*

None of the *S. dysgalactiae* isolates was negative for all resistance genes tested by PCR amplification. In summary, 1.1, 12.5, 18.2, 36.4, and 31.8% of isolates were positive for at least one tested resistance gene in 1, 2, 3, 4, or 5 antimicrobial drug classes (Figure 3). Of all 31 tested resistance genes, *blaTEM* (n = 75) was detected the most prevalent gene by PCR, followed by *tetM* (n = 66), *sul1* (n = 63), and *ermB* (n = 52; Table 5). A single isolate was positive for *blaSPM-1*, *mdfA/mefE*, *ermC*, *tetD*, and *gyrA*, whereas many other antimicrobial resistance genes were even not detected

although the isolates showed phenotypic resistance against antimicrobials in the specific antimicrobial drug class (Table 5). Table 6 depicts the prevalence of different phenotypically resistant *S. dysgalactiae* isolates exhibiting resistance to 8 different antimicrobial agents and the occurrence of different resistance genes among these isolates. Overall, 5 resistance genes (*aphA-1*, *aphA-2*, *aphA-3*, *aadA1/aadA2*, and *aad-6*) encoding for aminoglycoside resistance, 3 resistance genes (*blaTEM*, *blaIMP*, *blaSPM-1*) encoding for β-lactams, 4 genes (*ermA/TR*, *ermB*, *mdfA/mefE*, *ermC*) responsible for resistance to macrolides, 1 gene (*gyrA*) responsible for quinolone resistance, 1 gene (*rrs*) encoding for streptomycin resistance, 2 genes (*sul1*, *sul2*) encoding

Figure 3. Distribution of the number of genotypically resistant *Streptococcus dysgalactiae* isolates stratified by the number of antimicrobial drug classes of which at least one resistance gene of the corresponding class was amplified (i.e., 1, 2, 3, 4, or 5). 1 = the detected resistance gene(s) belonged to one antimicrobial drug class; 2 = the detected resistance genes belonged to 2 different antimicrobial drug classes; 3 = the detected resistance genes belonged to 3 different antimicrobial drug classes; 4 = the detected resistance genes belonged to 4 different antimicrobial drug classes; 5 = the detected resistance genes belonged to 5 different antimicrobial drug classes.

Table 5. Distribution of antimicrobial resistance genes detected in *Streptococcus dysgalactiae* isolates (n = 88) recovered from milk samples of bovine clinical mastitis cases collected on 17 dairy farms stratified by province

Antimicrobial resistance gene	No. of isolates carrying the specific antimicrobial resistance gene stratified by province					Total (n = 88)
	Guangdong (n = 3)	Hebei (n = 13)	Inner Mongolia (n = 68)	Shanghai (n = 1)	Shandong (n = 3)	
<i>aphA-1</i>	2	5	25	0	0	32
<i>aphA-2</i>	0	0	12	0	0	12
<i>aphA-3</i>	0	0	9	0	0	9
<i>aadA1/aadA2</i>	0	0	22	0	0	22
<i>aad-6</i>	0	0	14	0	0	14
<i>blaTEM</i>	3	9	60	1	2	75
<i>blaIMP</i>	1	0	15	0	0	16
<i>blaSPM-1</i>	0	0	1	0	0	1
<i>blaVIM</i>	0	0	0	0	0	0
<i>blaSHV</i>	0	0	0	0	0	0
<i>mecA</i>	0	0	0	0	0	0
<i>ermA/TR</i>	2	4	1	1	0	8
<i>ermB</i>	1	10	38	1	2	52
<i>mdfA/mefE</i>	0	1	0	0	0	1
<i>ermC</i>	0	0	1	0	0	1
<i>gyrA</i>	0	0	1	0	0	1
<i>parC</i>	0	0	0	0	0	0
<i>rrs</i>	1	0	3	0	0	4
<i>rpsL</i>	0	0	0	0	0	0
<i>sul1</i>	2	13	45	0	3	63
<i>sul2</i>	2	7	27	0	0	36
<i>sul3</i>	0	0	0	0	0	0
<i>tetA</i>	0	0	0	0	0	0
<i>tetB</i>	0	0	0	0	0	0
<i>tetC</i>	0	0	0	0	0	0
<i>tetD</i>	0	0	1	0	0	1
<i>tetE</i>	0	0	0	0	0	0
<i>tetK</i>	1	0	3	0	0	4
<i>tetL</i>	2	3	15	0	0	20
<i>tetM</i>	2	12	49	0	3	66
<i>tetO</i>	2	4	25	0	0	31

for sulfonamide resistance, and 5 genes (*tetD*, *tetK*, *tetL*, *tetM*, *tetO*) encoding for tetracycline resistance, in various frequencies detected as shown in Table 6.

Association Between Phenotypic and Genotypic Resistance Profiles

The survival analysis was performed only for those resistance genes that were carried by at least 2 *S. dysgalactiae* isolates (Table 7). For instance, a single isolate was found positive for *blaSPM-1*, *mdfA/mefE*, *ermC*, *tetD*, and *gyrA*; therefore, we did not perform the survival analysis for these resistance genes. For other genes, such as *blaTEM*, *blaIMP*, *blaTEM*, *blaIMP*, *ermA/TR*, *ermB*, *sul1*, *sul2*, *tetM*, *tetK*, and *tetO*, survival analysis was assessed. The results of survival analysis in all *S. dysgalactiae* isolates showed a significant association between the presence of *ermB* and survival at increasing erythromycin concentrations as shown in Figure 4 (log rank statistic, chi-squared 5.05, 1 df, $P = 0.02$). However, survival at increasing erythromycin concentrations did not differ between *ermA/TR* positive and negative isolates. Additionally,

survival at increasing cephalixin concentrations tended to be associated with the presence of the *blaIMP* gene (log rank statistic, chi-squared 3.6, 1 df, $P = 0.06$). Nevertheless, no significant associations were observed between the survival of the *S. dysgalactiae* isolates at increasing concentrations of other tested antimicrobial drugs and the presence of the specific resistance genes (Table 7).

DISCUSSION

This is the first comprehensive study to investigate both the phenotypic and genotypic antimicrobial resistance profiles of *S. dysgalactiae* bacteria originating from dairy cows that suffered from clinical mastitis in Chinese dairy farms. The generated data set was rather unique and allowed us to get better insights into the prevalence and the resistance of *S. dysgalactiae* as a cause of clinical mastitis in dairy cows in China because a large number of cows belonging to 74 different herds located in not less than 14 Chinese provinces were included, and as all milk samples were processed by 1 laboratory.

Table 6. Prevalence of phenotypically resistant *Streptococcus dysgalactiae* isolates stratified by antimicrobial drug that carried the tested antimicrobial resistance genes

Antimicrobial drug class	Antimicrobial resistance gene	No. (%) of isolates that showed antimicrobial resistance against the specified antimicrobial drug ¹							
		CX (n = 30)	CRO (n = 12)	E (n = 42)	NOR (n = 16)	K (n = 79)	S (n = 52)	SM (n = 68)	TE (n = 29)
Aminoglycosides	<i>aphA-1</i>	10 (33.3)	4 (33.3)	19 (45.2)	1 (6.3)	26 (32.9)	22 (42.3)	22 (32.4)	7 (24.1)
	<i>aphA-2</i>	6 (20.0)	4 (33.3)	8 (19.0)	1 (6.3)	11 (13.9)	5 (9.6)	8 (11.8)	3 (10.3)
	<i>aphA-3</i>	0 (0)	0 (0)	4 (9.5)	5 (31.3)	8 (10.1)	9 (17.3)	9 (13.2)	7 (24.1)
	<i>aadA1/aadA2</i>	8 (26.7)	4 (33.3)	11 (26.2)	2 (12.5)	20 (25.3)	11 (21.2)	18 (26.5)	7 (24.1)
	<i>aad-6</i>	6 (20)	1 (8.3)	6 (14.3)	6 (37.5)	13 (16.5)	11 (21.2)	14 (20.6)	7 (24.1)
β-Lactams	<i>blaTEM</i>	26 (86.7)	9 (75.0)	36 (85.7)	14 (87.5)	66 (83.5)	48 (92.3)	60 (88.2)	24 (82.8)
	<i>blaIMP</i>	16 (53.3)	3 (25.0)	7 (16.7)	7 (43.8)	15 (19.0)	13 (25.0)	14 (20.6)	9 (31.0)
	<i>blaSPM-1</i>	0 (0)	0 (0)	0 (0)	1 (6.3)	1 (1.3)	0 (0)	1 (1.5)	0 (0)
Macrolides	<i>ermA/TR</i>	0 (0)	0 (0)	2 (4.8)	1 (6.3)	7 (8.9)	6 (11.5)	7 (10.3)	0 (0)
	<i>ermB</i>	17 (56.7)	5 (41.7)	28 (66.7)	6 (37.5)	47 (59.5)	27 (51.9)	42 (61.8)	16 (55.2)
	<i>mdfA/mefE</i>	0 (0)	0 (0)	1 (2.4)	0 (0)	0 (0)	1 (1.9)	0 (0)	0 (0)
	<i>ermC</i>	0 (0)	0 (0)	1 (2.4)	0 (0)	0 (0)	1 (1.9)	0 (0)	0 (0)
Quinolones	<i>gyrA</i>	1 (3.3)	1 (8.3)	1 (2.4)	1 (6.3)	1 (1.3)	1 (1.9)	0 (0)	1 (3.4)
Streptomycin	<i>rrs</i>	1 (3.3)	1 (8.3)	0 (0)	1 (6.3)	3 (3.8)	3 (5.8)	4 (5.9)	1 (3.4)
Sulfonamides	<i>sul1</i>	23 (76.7)	6 (50)	32 (76.2)	9 (56.3)	56 (70.9)	1 (1.9)	55 (80.9)	23 (79.3)
	<i>sul2</i>	9 (30.0)	1 (8.3)	12 (28.6)	6 (37.5)	29 (36.7)	37 (71.2)	29 (42.6)	9 (31.0)
Tetracyclines	<i>tetD</i>	0 (0)	0 (0)	1 (2.4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	<i>tetK</i>	2 (6.7)	2 (16.7)	2 (4.8)	3 (18.8)	4 (5.1)	3 (5.8)	2 (2.9)	3 (10.3)
	<i>tetL</i>	5 (16.7)	2 (16.7)	9 (21.4)	3 (18.8)	16 (20.3)	12 (23.1)	16 (23.5)	6 (20.7)
	<i>tetM</i>	21 (70.0)	10 (83.3)	34 (81)	11 (68.8)	61 (77.2)	37 (71.2)	54 (79.4)	22 (75.9)
	<i>tetO</i>	13 (43.3)	5 (41.7)	15 (35.7)	3 (18.8)	28 (35.4)	18 (34.6)	26 (38.2)	11 (37.9)

¹CX = cephalexin; CRO = ceftriaxone; E = erythromycin; NOR = norfloxacin; K = kanamycin; S = streptomycin; SM = sulfonamide; TE = tetracycline.

In the current study, the prevalence of *S. dysgalactiae* causing clinical mastitis was relatively lower than the other reported studies; however, a large variation among herds within the same province was observed. Approximately 7.5% of the tested milk samples was found to be culture positive for *S. dysgalactiae*. The isolation rate was much lower than the 72.3% isolation of *S. dysgalactiae* from bulk milk samples (Bi et al., 2016). This considerable difference in the prevalence of *S. dysgalactiae* might at least partly be explained by the fact that bulk milk samples not only contain bacteria present in the milk of infected quarters, but

might also be contaminated by bacteria coming from the milking equipment and environment (Elmoslemany et al., 2009a,b). Still, the prevalence of *S. dysgalactiae* from clinical mastitis in our study was consistent with the prevalence of 10.5% from clinical mastitis in China (Gao et al., 2017), 12.1% from clinical mastitis in Norway (Whist et al., 2007), and 4.0% from clinical mastitis in Thailand (Leelahapongsathon et al., 2014).

Antimicrobial susceptibility was determined using serial dilutions and determination of the minimal inhibitory concentrations instead of common agar diffusion assays. It might be helpful to adjust the thera-

Table 7. Cox proportional hazard model describing the association between bacterial survival with increasing concentrations of different antimicrobial drugs and the presence of the specific antimicrobial resistant genes

Antimicrobial drug	Resistance gene	β^1	SE	HR ²	95% CI ³	P-value
Cephalexin	<i>blaTEM</i>	-0.002	0.329	0.998	0.523-1.903	0.99
	<i>blaIMP</i>	0.444	0.234	1.559	0.986-2.464	0.06
Ceftriaxone	<i>blaTEM</i>	-0.266	0.248	0.766	0.471-1.245	0.28
	<i>blaIMP</i>	0.191	0.203	1.210	0.812-1.803	0.35
Erythromycin	<i>ermA/TR</i>	-0.236	0.414	0.790	0.351-1.776	0.57
	<i>ermB</i>	0.480	0.213	1.615	1.063-2.454	0.02
Sulfonamide	<i>sul1</i>	0.039	0.264	1.040	0.620-1.744	0.88
	<i>sul2</i>	0.238	0.255	1.268	0.769-2.093	0.35
Tetracycline	<i>tetM</i>	-0.179	0.243	0.836	0.520-1.345	0.46
	<i>tetK</i>	0.570	0.423	1.769	0.772-4.053	0.18
	<i>tetO</i>	-0.105	0.156	0.901	0.664-1.222	0.50

¹ β = regression coefficient.

²HR = hazard ratio.

³95% CI = the reference is the isolates without the gene or the ones with the gene.

Figure 4. *Streptococcus dysgalactiae* survival curve in varying concentrations of erythromycin. Survival of bacteria without (dashed line) or with (solid line) PCR-based detection of *ermB* in different concentrations of erythromycin. With the increasing concentration of erythromycin, the survival probability of *S. dysgalactiae* without (dashed line) and with (solid line) PCR-based detection of *ermB* showed a significant difference.

peutic doses and to achieve effective concentrations of these drugs. Survival analysis was used to describe the association between detection of genes encoding resistance compounds and the respective antimicrobials. Survival analysis can help us to investigate the variations in the growth of *S. dysgalactiae* with the complete range of the tested antimicrobials (Sampimon et al., 2011).

The panel of antimicrobial agents evaluated in this study included those antimicrobials that are routinely used on Chinese dairy farms to treat bovine mastitis and other infectious diseases. The antimicrobial agents to which antimicrobial resistance was demonstrated most frequently were kanamycin (89.8%) and sulfonamide (83.0%), followed by streptomycin (58.0%), erythromycin (47.7%), and tetracycline (33.0%). This is not that surprising as kanamycin and streptomycin are mainly active against gram-negative bacteria and *S. dysgalactiae* belongs to the group of gram-positive bacteria. An intrinsic resistance for most of *Streptococcus* spp. has also been described for sulfonamide and aminoglycosides (Swedberg et al., 1998; Porter and Kaplan, 2011). In contrast, in a study of bovine *S. dysgalactiae* isolates from New Zealand, the resistance to trimethoprim/sulfamethoxazole was only 17% (McDougall et al., 2014). The latter discrepancy between our results and those obtained by McDougall et al. might be hypothetically explained by the fact that the tested molecule was a combination of trimethoprim/sulfamethoxazole, whereas in our study sulfonamide was tested as a single compound. In a recent study,

Lu et al. (2016) also found a high level of antimicrobial resistance against erythromycin, clindamycin, and tetracycline in 56 *S. dysgalactiae* subspecies *equisimilis* isolates that were isolated from humans recovering from infectious diseases in 2 territorial hospitals in Beijing, China (Lu et al., 2016). Similar findings were recently observed in Taiwan (Lo et al., 2015) and Austria (Leitner et al., 2015). Strikingly, in the current study some of the clinical isolates from bovine mastitis were found to be resistant to cephalexin (34.1%) and ceftriaxone (13.6%; i.e., first- and third-generation cephalosporins, respectively). This finding is somewhat surprising as resistance of *S. dysgalactiae* against these antimicrobial agents has not been reported before in human or veterinary medicine. The observed increasing level of resistance against the β -lactam antimicrobials might at least partly be explained by the increasing use of cephalosporins, more specifically of the third-generation cephalosporin ceftiofur, for the treatment of infectious diseases on Chinese dairy farms (Ali et al., 2016). The increasing use of β -lactam antimicrobials might have resulted in a selective pressure toward the more resistant isolates.

The phenotypic antimicrobial resistance profiles of the recovered *S. dysgalactiae* isolates not only differed between herds but also within herds. The latter observation strongly indicates that the isolates causing clinical mastitis on one single farm may belong to different strain types. On the other hand, in one herd, the same phenotypic antimicrobial resistance pattern was observed in 7 *S. dysgalactiae* isolates recovered from 7

different clinical mastitis cases, suggesting the presence of a common infection source. The latter finding could be indicative for the contagious spread of *S. dysgalactiae* between cows during milking via teat liners, hands, and so on (Lundberg et al., 2014). Still, because no molecular strain-typing was performed via sophisticated techniques such as pulsed field gel-electrophoresis or multi-locus sequencing typing, caution is needed in the interpretation of this finding.

In *S. dysgalactiae* isolates carrying *blaTEM* or *blaIMP* gene, the prevalence of phenotypic resistance against cephalixin was higher than against ceftriaxone. Only 12 of 30 isolates that showed resistance against cephalixin were also found to be resistant against ceftriaxone. Ceftriaxone belongs to the third generation of cephalosporin, and it was introduced to the market more recently and has not been used that long compared with first-generation cephalosporins including cephalixin. The latter finding might stress the positive relationship between the antimicrobial drug use and the spread of antimicrobial resistance within a population.

Interestingly, all *S. dysgalactiae* isolates carried at least one resistance gene, whereas only a small number of *S. dysgalactiae* isolates showed phenotypic resistance against the associated antimicrobial. Although a trend toward a positive association between the presence of the *blaTEM* gene and bacterial survival was observed, comparison of survival curves for isolates with and without resistance genes to cephalixin and ceftriaxone also showed that several *bla*-gene positive isolates did not survive at low cephalixin or ceftriaxone concentrations (or both). This intriguing finding might be due to the lack of gene expression without induction as has already been described for *Staphylococcus aureus* (De Oliveira et al., 2000) or lack of functionality (Rahman et al., 2005). Conversely, 16 isolates were found to be phenotypically resistant to norfloxacin, whereas only one single isolate was detected to carry *gyrA*. The latter finding might be explained by mutation in the primer binding sites of resistance genes or the existence of not yet identified resistance genes (Haveri et al., 2005). Clearly, phenotypic resistance is not inevitably indicative for the presence of resistance genes, and vice versa. A similar discrepancy was reported by Sampimon et al. (2011) in which the phenotypic and genotypic antimicrobial resistance of bovine CNS isolates was investigated. A larger data set is needed to unequivocally determine the association between the presence of antimicrobial resistance genes and the phenotypic resistance patterns. Comprehensive consideration of the genotypic resistance and phenotypic resistance profiles could make the use of medication in case of clinical disease safer, more effective, and less costly.

CONCLUSIONS

The prevalence of *S. dysgalactiae* isolates recovered from milk samples of bovine mastitis cases and their antimicrobial resistance profiles considerably differ between and within dairy herds in China. Survival analysis showed a significant association between the presence of *ermB* and survival of the *S. dysgalactiae* isolates at increasing erythromycin concentrations. However, no other statistically significant associations were observed between the phenotypic and genotypic resistance profiles. The considerable variations in the prevalence and phenotypic and genotypic antimicrobial resistance profiles among Chinese dairy herds strongly suggest the need for antimicrobial susceptibility surveillance at the herd level to ensure optimal treatment results.

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