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ORIGINAL RESEARCH

Antimicrobial Resistance and Virulence Factor of *Streptococcus dysgalactiae* Isolated from Clinical Bovine Mastitis Cases in Northwest China

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Objective: *Streptococcus dysgalactiae* is a major pathogen in bovine mastitis. The purpose of this study was to survey the prevalence, antimicrobial resistance, as well as the spread of resistance and virulence-associated gene of *S. dysgalactiae.*

Methods: A total of 60 *S. dysgalactiae* strains were obtained from 830 milk samples from Holstein cows with clinical mastitis. Antimicrobial resistance was examined by the disk diffusion method. Antimicrobial resistance and virulence genes were investigated by PCR, agarose gel electrophoresis and 16S rRNA gene sequencing.

Results: All isolates were resistant to tetracycline and showed a high level of resistance to aminoglycoside antibiotics, where 81.67% of the strains were multi-resistant to these ten sorts of antibiotics. In addition, the most prevalent resistance gene in *S. dysgalactiae* was *aphA-1* (98.33%), followed by *blaTEM* (96.67%), *ermB* (83.3%), *aadA1/aadA2* (78.33%) and *tetL* (73.33%). Totally, seven virulence genes with 25 combination patterns were detected in these isolates, and each isolates harbored at least one virulence gene. 21.67% of the isolates carried three or more virulence genes, while one strain with seven virulence-related genes and belonged to *cfb+lmb+eno+napr+bca+scpB+cyl*.

Conclusion: These findings indicate that *S. dysgalactiae* isolated from clinical bovine mastitis cases in Northwest China show a variety of molecular ecology and are highly resistant to antibiotics commonly used in dairy farms. This research will help investigators better understand the pathophysiology *S. dysgalactiae* in bovine mastitis and choose the appropriate antibiotics to treat mastitis.

Keywords: *Streptococcus dysgalactiae*, bovine mastitis, antimicrobial resistance, virulence gene

Introduction

Bovine mastitis is one of the most prevalent and costly diseases concerning the dairy industry worldwide.[1] It is a kind of topical inflammation reaction, mainly due to the invasion of mammary gland tissue by microorganism.[2,3] *S. dysgalactiae* has become the major cause behind several mammalian infections, which can lead to streptococcal mastitis/endometritis in domestic mammals and skin lesions, meningitis, and bacteremia in humans.[4] In Swedish and other countries, it is recognized as the most common causative pathogens of bovine mastitis.[5–7] Besides, *S. dysgalactiae* is generally considered as an environmental pathogen. Although major advances have been seen in some prevent procedures of preventing and controlling mastitis, it exhibits less effective against the environmental pathogens, which produces more difficulty to control *S. dysgalactiae*.[8–10]

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Antimicrobial agents have always been used to prevent and control of mastitis around the world.[11,12] However, the abuse of antibiotics leads to antimicrobial resistance among causative agents and causes reactions in humans allergic to antimicrobials. This phenomenon is becoming more and more serious, which is coming to our notice. Previous investigations have confirmed that *S. dysgalactiae* possesses phenotypic and genotypic resistance to some common antimicrobial agents, such as Kanamycin (*aphA-1, aphA-2, aphA-3, aadA1/aadA2, aad-6*), β-lactam antibiotics (*blaTEM, blaIMP, blaSPM-1*), erythromycin (*ermA, ermB, ermC, mefE*), streptomycin (*rrs*), tetracyclines (*tetD, tetK, tetL, tetM, tetO*), etc.[13,14] The virulence-associated determiners of *S. dysgalactiae* play a crucial role in the pathogenesis of the causative agents, including α-enolase, nephritis-associated plasminogen-binding receptor, β- hemolysin, Lamining-binding Protein.[14–16] But in fact, little is known about these characteristics about the *S. dysgalactiae* isolated from bovine mastitis in Northwest, China. Therefore, the aim of this study is to investigate the antimicrobial resistance, resistance genes and virulence genes of it.

Materials and Methods

Sample Collection

Eight hundred and thirty (830) clinical mastitis milk samples were aseptically collected between 2016 and 2019. Herds selected in this study were from large-scale commercial dairy farms with good breeding administration measures; Mechanized Milking System is adopted by all the cattle farms. Each farm has 2000–3000 livestock with a minimum of 500 lactating Holstein-Friesian cows, (Ningxia Prov. = 342 samples from 10 farms, Gansu Prov. = 196 samples from 7 farms, Xinjiang Prov. = 170 samples from 6 farms, Shaanxi Prov. = 122 samples from 4 farms). The incidence of bovine mastitis in cattle farm ranges from 2% to 10%, and the parity of sick cows is about 4 to 5. In Northwest China, the incidence is higher from January to February and from June to September, so the samples were mainly collected during this period.

The clinical mastitis was confirmed by the California Mastitis Test (CMT). All of these farms employed veterinarians who had received professional training on sampling procedures and aseptic techniques for collecting samples. After sampling, the veterinarians disinfected the breasts of dairy cows with 75% ethanol and milk samples were transported to the laboratory at 4 °C for microbiological culture.

The sampling process was similar to normal commercial milking and met the requirements of animal welfare. This study does not involve animal experiments therefore ethical approval for this study was not needed.

Microbiological Culture and Identification

A volume of 20 μL of each sample was plated on sheep blood agar (Huan kai, Guangdong, China) at 37 °C aerobically for 24 to 48 h. It was considered as cultured positive if 1 or more colonies were observed. Milk samples with 3 or more species were considered contaminated, unless *Staphylococcus aureus* or *Streptococcus agalactiae* were isolated.[7] After growth, every single colony with different morphology was sub-cultured on blood agar. Another optional sub-culture was conducted if different morphological colonies grew on the same plate. A single colony was enrichment cultured in nutrient broth at 37 °C for 24 to 48 h and stored with 15% glycerol at −80 °C.

The colony was cultured in 2 mL of Tryptone Soya broth (TSB; Oxoid, UK) at 37 °C for 24–48 h. Then, the genomic DNA was extracted using the Bacterial DNA Kit (Omega, USA) following the manufacturer's protocol. Next, 16S rRNA gene sequencing (Tsingke, Xi'an, China) was used to identify the strains by the PCR amplified products of the extracted DNA.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility against 13 antimicrobial agents were determined by disc diffusion method on Mueller-Hinton agar (MHA; Oxoid, United Kingdom) supplemented with 5% sheep blood (Solarbio, Beijing, China) according to the Clinical and Laboratory Standards Institute, and E-test detected meropenem.[17] The commercially available discs (Oxoid, United Kingdom) used in this study included cefepime (30 μg), cefotaxime (30 μg), vancomycin (30 μg), erythromycin (15 μg), tetracycline (30 μg), levofloxacin (5 μg), chloramphenicol (30 μg), clindamycin (2 μg), linezolid (30 μg), kanamycin (30 μg), gentamycin (10 μg), streptomycin (10 μg), sulphamethoxazole (23.75 μg). E-test strip (Liofulchem, Italy) of meropenem ranged from 0.016 μg/mL to 256 μg/mL. For those which did not have a reference breakpoint of resistance to *Streptococcus* spp., the resistant breakpoints referred to either an antimicrobial of the same antimicrobial drug class or another pathogen group.[13]

Antimicrobial Resistance and Virulence Genotyping

The antimicrobial resistance and virulence genes were tested by simplex PCR amplification. Most resistance genes were from references except for vancomycin-related and linezolid-related resistance genes were designed by Primer 5.0 software. The detailed information of the primers is shown in Tables 1 and 2. All primers were synthesized by Tsingke Biological Technology (Xi'an, China). The system for detecting resistance and virulence genes was 25 μL, consisting of 22 μL T3 Super PCR Mix (Tsingke, Xi'an, China), 1 μL DNA sample material, 1μL forward, and 1μL reverse primers. The reaction following the manufacturer's protocol and run in a thermal cycler (Biometra T Advanced, German). The PCR products (5 μL) were analyzed by electrophoresis on 1% agarose gel and stained with Gel-red (Tsingke, Xi'an, China). The results were visualized and photographed by a UV transilluminator. In addition, DNA sequencing (Tsingke, Xi'an, China) identify was used to further identify the PCR amplified products of the resistance and virulence genes, and the gene sequence was compared in the NCBI gene bank. Hence, electrophoresis and DNA sequencing technique were used to determine whether the strain carried the corresponding resistance and virulence gene.

Results

Isolation and Identification of *S. dysgalactiae*

In this study, the similarity among the sequencing results of all isolates was ≥99%. In all the milk samples, a total of 91 samples have no bacteria growth, which were regard as cultured negative. Except that, 31 samples were defined as contaminated. The rest of 708 milk samples were cultured positive, including *Escherichia coli* (143/830, 17.23%), *Klebsiella spp*. (76/830, 9.15%), *Coagulase negative staphylococci* (68/830, 8.2%), *S. agalactiae* (72/830, 8.7%), *S. aureus* (113/830, 13.61%), *S. dysgalactiae* (60/830, 7.23%), *Enterobacter spp*. (53/830, 6.39%), *Streptococcus uberis* (21/830, 2.53%), *Pseudomonas spp*. (32/830, 3.86%), *Trueperella pyogenes* (17/830, 2.05%), *Aerococcus viridans* (23/830, 2.78%) and mixed-culture (30/830, 3.61%).

Antimicrobial Susceptibility Testing

The phenotypic resistance of fourteen antimicrobial of *S. dysgalactiae* is shown in Table 3. The resistance rate of tetracycline was the highest, up to 100%, followed by aminoglycosides (greater than 70%). Each of the resistance rates of chloramphenicol, erythromycin, sulphamethoxazole, levofloxacin, vancomycin, linezolid, cefotaxime, and cefepime was less than half, only 33.33%, 36.67%, 18.33%, 13.33%, 46.67%, 20%, 45%, 11.67%, respectively. In addition, all isolates were susceptible to meropenem. Notably, 81.67% of the strains were multidrug-resistant to these 14 antibiotics, and 2 isolate strains were only sensitive to linezolid and meropenem.

Genotypic Resistance Profiles of *S. dysgalactiae*

The sequencing results of all resistance gene were 100% similar. The resistance gene bands were clearly recorded according to the electrophoresis results, which revealed the same results as DNA sequencing. As shown in Table 3, the most frequently detected antimicrobial resistance gene was *aphA-1* (98.33%), followed by *blaTEM* (96.67%), *ermB* (83.33%), *aadA1/aadA2* (78.33%), *tetL* (73.33%), *aphA-3* (65%), *cat1* (63.33%), *cat2* (68.33%), *tetS* (45%). Moreover, tetracyclines resistance genes (*tetD*, 11.67%; *tetM*, 8.33%; *tetO*, 10%), erythromycin resistance genes (*mefE*, 10%), the gene of *aad6* (3.33%) about aminoglycosides, and the gene of *blaSHV* (1.67%) about β-Lactam antibiotics were also amplified. Additionally, *vanA* (6.67%), *vanB* (8.33%), *vanC1/C2* (1.67%) resistance genes related to vancomycin, and *optrA* (1.67%), *poxtA* (25%) related to linezolid were detected as well. However, none of the stains were positive for the resistance genes related to the tetracyclines (*tetK*), aminoglycosides (*aphA-2*), erythromycin (*ermA, ermC*), vancomycin (*VanC2/C3*), and linezolid (*Cfr*). Furthermore, the *rpsL* and *rrs* for streptomycin resistance gene were not detected in any of the strains.

Genotypic Virulence Profiles of *S. dysgalactiae*

All virulence gene sequencing results were 100% similar. The result of agarose gel electrophoresis was corresponded with the DNA sequencing. The results showed that all isolates carried *napr*, and 36.67% carried the gene *cfb*. The genes *eno, lmb, bca, scpB* and *cyl* were detected as well, in 16.67%, 3.33%, 6.67%, 18.33% and 13.33% of the isolates, respectively. However, the *bac* gene was negative in any isolate. In this study, all tested isolates had at least one virulence gene, and 21.67% of those harbored three virulence-associated genes, and one isolate carried seven genes.

Table 1 Target Resistance Gene Information

Antimicrobial Drug Class	Target Gene	Primer Sequence (5′→3′)	Product Size (bp)	References
Tetracyclines	*tetD*	ATTACACTGCTGGACGCGAT CTGATCAGCAGACAGATTGC	1104	Zhang et al.[13]
	tetK	GTAGGATCTGCTGCATTCCC CACTATTACCTATTGTCGC	155	Zhang et al.[13]
	tetL	TGGTGGAATGATAGCCCATT CAGGAATGACAGCACGCTAA	229	Zhang et al.[13]
	tetM	GTGGAGTACTACATTTACGAG GAAGCGGATCACTATCTGAG	359	Zhang et al.[13]
	tetO	ACGGARAGTTTATTGTATACC TGGCGTATCTATAATGTTGAC	171	Zhang et al.[13]
	tetS	GAAAGCTTA CTATACAGTAGC AGGAGTATCTACAATATTTAC	229	Zhang et al.[47]
Macrolides	*ermA*	TCAGGAAAAGGACATTTTACC ATACTTTTTGTAGTCCTTCTT	432	Zhang et al.[13]
	ermB	ATTGGAACAGGTAAAGGGC GAACATCTGTGGTATGGCG	442	Zhang et al.[47]
	ermC	TCAAAACATAATATAGATAAA GCTAATATTGTTTAAATCGTCAA	642	Zhang et al.[13]
	mefE	AGTATCATTAATCACTAGTGC TTCTTCTGGTACTAAAAGTGG	348	Zhang et al.[13]
Aminoglycosides	*aphA-1*	ATGGGCTCGCGATAATGTC CTCACCGAGGCAGTTCCAT	600	Zhang et al.[13]
	aphA-2	GAACAAGATGGATTGCACGC GCTCTTCAGCAATATCACGG	680	Zhang et al.[13]
	aphA-3	GGGGTACCTTTAAATACTGTAG TCTGGATCCTAAAACAATTCATCC	848	Zhang et al.[13]
	aadA1/aadA2	GCAGCGCAATGACATTCTTG ATCCTTCGGCGCGATTTTG	282	Zhang et al.[13]
	aad-6	AGAAGATGTAATAATATAG CTGTAATCACTGTTCCCGCCT	978	Zhang et al.[13]
Streptomycin	*rpsL*	GGCCGACAAACAGAACGT GTTCACCAACTGGGTGAC	501	Zhang et al.[13]
	rrs	GAGAGTTTGATCCTGGCTCAG TGCACACAGGCCACAAGGGA	1042	Zhang et al.[13]
Phenicols	*cat1*	CTTGTCGCCTTGCGTATAAT ATCCCAATGGCATCGTAAAG	508	Tian et al.[14]
	cat2	AACGGCAYGATGAACCTGAA ATCCCAATGGCATCGTAAAG	547	Tian et al.[14]
β-Lactams	*blaIMP*	CTACCGCAGCAGAGTCTTTG AACCAGTTTTGCCTTACCAT	587	Zhang et al.[13]

(*Continued*)

Table 1 (Continued).

Antimicrobial Drug Class	Target Gene	Primer Sequence (5′→3′)	Product Size (bp)	References
	blaSHV	ATGCGTTATATTCGCCTGTG TTAGCGTTGCCAGTGCTCGA	860	Zhang et al.[13]
	blaSPM-1	CCTACAATCTAACGGCGACC TCGCCGTGTCCAGGTATAAC	649	Zhang et al.[13]
	blaTEM	ATGAGTATTCAACATTTTCGTG TTACCAATGCTTAATCAGTGAG	860	Zhang et al.[13]
	blaVIM	ATTCCGGTCGGAGAGGTCCG GAGCAAGTCTAGACCGCCCG	633	Zhang et al.[13]
	mecA	TGGCTATCGTGTCACAATCG CTGGAACTTGTTGAGCAGAG	310	Zhang et al.[13]
Glycopeptides	*vanA*	TTCAGGCTCATCCTTCGG TCCACCTCGCCAACAACT	174	
	VanB	TGAGCAGCAAATCCACAA TCGCCTTCAATTACATCG	210	
	VanC1/C2	TGCCTTATGTTGGTTGCC TGGTGCTGGGACAGTGAT	494	
	VanC2/C3	TGACAAATCAAGCCAACC GCACTGCGGAACAATAAG	172	
Oxazolidinones	*Cfr*	TATGGGAATGGGAGAAGC AGGAGAACTGACGGTTGG	436	
	optrA	GGTGGTCAGCGAACTAAG CGTTCAATCAAGCGTGTA	341	
	poxtA	ATAAGGTCGGTATTGTCG TCTGCCTCATAGAAGTCG	325	

Discussion

S. dysgalactiae is one of the most ubiquitous *Streptococcus* species, invading mammary glands when appropriate conditions permit their activities.[15] In this study, the proportion of *S. dysgalactiae* isolated from CM was 7.23%. The results for the prevalence of *S. dysgalactiae* were similar to those of Finland, South Eastern Ethiopia, France and Portugal, ranging from 5.10%-8.80%, but lower than a national epidemiological study in China.[7,18–21] The prevalence difference may be due to sampling number, geographical location, season, and management strategies. Effective medication, monitoring the development of resistant and virulent strains in certain ecological niches, and detecting the antimicrobial susceptibility profiles and virulence factors of *S. dysgalactiae* are crucial for preventing and treating the bovine mastitis caused by this pathogen.[15,22]

This study found that the isolates showed high resistance to tetracycline, followed by streptomycin, kanamycin, gentamycin and clindamycin, similar to a Portuguese study.[21] According to the permission of prescription drug in veterinary based on Announcement No.1997 of the Ministry of Agriculture of the People's Republic of China, many kinds of antimicrobials were permitted to use as antimicrobial drugs in veterinary medicine in China.[23] Long-term and widespread use of antibiotics to treat bacterial infection will definitely enhance the occurrence of multidrug-resistant *streptococcus* isolates, which would have more chance to cause bovine mastitis in the same dairy farm. The tetracycline testing results are similar to a Chinese report but much higher than one study in China, in which 59% *Streptococcus spp*. isolates are resistant to tetracycline.[14,24] This phenomenon may be due to the

Table 2 Target Virulence Gene Information

Function Protein	Tar-Get Gene	Primer Sequence (5′→3′)	Prodict Size (bp)	References
α-enolase	*eno*	ATGTCAATTATTACTGATGT CTATTTTTTTAAGTTATAGA	1308	Kaczorek et al.[34]
Nephritis-associated plasminogen-binding receptor	*napr*	GTTAAAGTTGGTATTAACGGT TTGAGCAGTGTAAGACATTTC	963	Kaczorek et al.[34]
CAMP factor	*cfb*	ATGGGATTTGGGATAACTAAGCTAG AGCGTGTATTCCAGATTTCCTTAT	193	Tian et al.[14]
Lamining-binding Protein	*lmb*	ACCGTCTGAAATGATGTGG GATTGACGTTGTCTTCTGC	572	Tian et al.[14]
C α protein	*bca*	TAACAGTTATGATACTTCACAGAC ACGACTTTCTTCCGTCCACTTAGG	535	Tian et al.[14]
C β protein	*bac*	TGTAAAGGACGATAGTGTGAAGAC CATTTGTGATTCCCTTTTGC	530	Tian et al.[14]
Streptococcal C5aïpeptidase- adhesion	*scpB*	CCAAGACTTCAGCCACAAGG CAATTCCAGCCAATAGCAGC	591	Tian et al.[14]
β-haemolisin	*cyl*	ACGGCTTGTCCATAGTAGTGTTTG AACGACACTGCCATCAGCAC	345	Tian et al.[14]

different sampling area and other *streptococci* resistance have been counted in their study. Conventionally, *S. dysgalactiae* has a high-level resistance to aminoglycoside antibiotics, because it is mainly used to treat gram-negative bacterial infection. The report implies that the resistance to commonly used antibiotics in dairy cow diseases of *S. dysgalactiae* isolated from dairy cow in China is seriously. We also find that 45% *S. dysgalactiae* isolates are not sensitive to cefotaxime. The levels observed in this investigation are 10% higher than those observed by Zhang et al and Tian et al[13,14] This may be due to the long-term and widespread use of β-lactam antimicrobials in this area. It is worth noting that vancomycin is considered the last line of defense against severe infections caused by gram-positive bacteria.[25] Strikingly, we found the *S. dysgalactiae* show resistance to vancomycin and linezolid for the first time. In addition, 81.67% of the strains are multi-drug-resistant. This sensational result has to be noticed because it may be a highly resistant "superbug".

This study also detected the corresponding resistance genes. It was found that the proportion of resistance genes related to aminoglycosides was at a high level, which had plenty of potentials, leading to the high resistance rate of the antibiotic. Although all strains were tetracycline-resistant, the related-resistant gene was only found in 76.67% of the strains. Inversely, a few were showed resistance to erythromycin, but most of them harbored the corresponding resistance gene. This rather contradictory result may be due to the absence of gene expression, mutations in the ribosomal target or some resistance genes that have not been identified yet.[26–28] These results suggest that phenotypic resistance is not necessarily related to resistance genes. In addition, one interesting finding was that *blaSHV* gene, vancomycin (*vanA, vanB, VanC1/C2*) and linezolid (*optrA, poxtA*) relevant genes were detected for the first time in *S. dysgalactiae* isolated from bovine mastitis. Since *S. dysgalactiae* is considered as a latent emerging zoonotic pathogen, this research output consider a phenomenon where public health security may be threatened.[29]

Antimicrobials are sometimes effective in vitro but ineffective when in vivo because of some invasiveness factors in the bacteria.[30] Many virulence factors of *Streptococcus* are involved in the infection and colonization of host cells and the escape of the immune system.[31] Genes *napr* and *eno* encoding binding host plasminogen protein, contribute to infect and colonize the host.[31,32] Plasminogen recruitment to the bacterial surface has been reported as a key pathogenic mechanism to promote bacterial adhesion to cell surface.[33] All isolates in this

Table 3 Phenotypic and Genotypic Characteristics of *S. dysgalactiae* Isolates

Strain Number	Resistance Pattern	Resistance Genes	Virulence Genes
1	TET+STR+KAN+CHL+VAN	*tetD+tetL+ermB+aphA-1+aphA-3+aadA1/aadA2+blaTEM*	*cfb+eno+napr*
2	TET+STR+KAN	*tetL+ermB+aphA-1+aphA-3+aadA1/aadA2+cat2+blaTEM*	*cfb+eno+napr*
3	TET+STR+KAN+VAN	*tetL+ermB+aphA-1+aphA-3+aadA1/aadA2+cat2+blaTEM*	*cfb+napr+cyl*
4	TET+STR+KAN	*tetD+tetL+ermB+aphA-1+aphA-3*	*naprI*
5	TET+STR+KAN+GEM+CLI+VAN+CTX	*tetL+ermB+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaTEM*	*cfb+napr*
6	TET+STR+KAN+GEM+CLI+CHL+CXT+VAN	*tetL+ermB+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaTEM*	*cfb+eno+napr+cyl*
7	TET+STR+KAN+GEM+CLI+VAN+CTX	*tetD+tetL+ermB+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaTEM*	*cfb+eno+napr+cyl*
8	TET+STR+KAN+GEM+CLI+CHL+ERY+VAN+LZD+CTX	*tetD+tetL+ermB+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaTEM+vanB+poxtA*	*eno+napr*
9	TET+STR+K+GEM+CHL+SSS+LEV+LZD	*tetL+ermB+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaTEM+poxtA*	*cfb+napr*
10	TET+STR+KAN+CLI+ERY	*tetD+tetL+ermB+aphA-1+aphA-3+aadA1/aadA2+aad-6+cat1+blaTEM*	*cfb+napr+cyl*
11	TET+STR+KAN+CLI+ERY	*tetL+tetM+tetS+ermB+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaTEM*	*cfb+napr*
12	TET+STR+KAN+GEM+CLI+ERY	*tetD+tetL+tetO+tetS+ermB+aphA-1+aadA1/aadA2+cat1+cat2+blaTEM*	*cfb+napr*
13	TET+STR+KAN+GEM	*tetL+tetS+ermB+aphA-1+aphA-3+aadA1/aadA2+cat2+blaTEM*	*cfb+napr*
14	TET+STR+KAN+CLI+ERY	*tetL+tetM+tetS+ermB+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaTEM*	*napr*
15	TET+GEM	*tetL+tetS+ermB+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaTEM*	*cfb+napr*
16	TET+STR+KAN+GEM+CLI+ERY	*tetL+tetO+tetS+ermB+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaTEM*	*cfb+napr*
17	TET+STR+KAN+CHL+LEV+SSS+CTX	*tetL+tetS+ermB+aphA-1+aphA-3+aadA1/aadA2+cat2+blaTEM*	*cfb+napr*
18	TET+STR+KAN	*tetD+tetL+ermB+aphA-1+aphA-3+cat1+cat2+blaTEM*	*napr+cyl*
19	TET+STR+KAN+GEM+CHL+VAN+LZD	*ermB+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaTEM+vanB+vanC1/C2*	*cfb+eno+napr*
20	TET+STR+KAN	*tetL+tetS+ermB+aphA-1+aphA-3+aadA1/aadA2+cat2+blaTEM*	*cfb+eno+napr*
21	TET+STR+KAN+GEM+CHL+VAN+LZD+CTX	*tetL+tetS+ermB+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaTEM+vanB*	*cfb+eno+napr+scpB+cyl*
22	TET+STR	*tetL+tetS+ermB+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaTEM*	*napr+scpB*
23	TET+STR	*tetL+tetS+ermB+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaTEM*	*cfb+napr+scpB*

(*Continued*)

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Table 3 (Continued).

Strain Number	Resistance Pattern	Resistance Genes	Virulence Genes
24	TET+STR+KAN	*tetL+ermB+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaTEM*	*cfb+napr*
25	TET+STR+KAN+GEM+SSS+CTX	*tetL+tetS+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaTEM*	*cfb+eno+napr+scpB*
26	TET+STR+GEM	*tetL+tetS+ermB+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaTEM*	*cfb+lmb+eno+napr+bca+scpB+cyl*
27	TET+STR+KAN+CLI	*tetL+tetS+ermB+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaSHV+blaTEM*	*cfb+lmb+napr+scpB+cyl*
28	TET+STR+KAN+CLI+VAN+CTX	*tetL+tetM+tetS+ermB+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaTEM*	*napr+scpB*
29	TET+STR+KAN+CLI+CHL+VAN+CTX	*tetL+tetS+ermB+aphA-1+aphA-3+aadA1/aadA2*	*napr+scpB*
30	TET+STR+KAN+GEM+CLI+ERY+VAN+LZD+CTX	*tetL+tetS+ermB+aphA-1+aphA-3+aadA1/aadA2+blaTEM*	*napr+scpB*
31	TET+STR+KAN+GEM+CLI+CHL+VAN+CTX	*tetL+tetS+ermB+aphA-1+aphA-3+aadA1/aadA2+cat1+blaTEM*	*napr+scpB*
32	TET+STR+KAN+GEM+CLI+CHL+SSS+LEV+ERY+LZD+CTX+FEP	*tetL+tetS+ermB+aphA-1+aphA-3+aadA1/aadA2+cat1+blaTEM+poxtA*	*napr+scpB*
33	TET+STR+KAN+GEM+CLI+CHL+ERY+SSS+LEV+VAN+CTX+FEP	*tetL+ermB+mefE+aphA-1+aadA1/aadA2+cat1+cat2+blaTEM+poxtA*	*napr+scpB*
34	TET+STR+KAN+CLI+CHL	*tetL+tetS+ermB+aphA-1+aphA-3+aadA1/aadA2+cat1+blaTEM+poxtA*	*napr*
35	TET+STR+KAN+GEM+CLI +ERY+SSS	*tetL+tetS+ermB+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaTEM*	*napr*
36	TET+STR+KAN+GEM+CLI +ERY+VAN	*tetL+tetS+ermB+mefE+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaTEM+vanA+poxtA*	*napr*
37	TET+STR+KAN+GEM+CLI+CHL+VAN+LZD+CTX	*tetL+tetS+mefE+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaTEM+poxtA*	*napr*
38	TET+STR+KAN+GEM+CLI+CHL+ERY+VAN+LZD+CTX+FEP	*tetL+tetM+tetS+aphA-1+aadA1/aadA2+cat2+blaTEM+poxtA*	*napr*
39	TET+STR+KAN+GEM+CLI+CHL+ERY+VAN+CTX+FEP	*tetL+tetS+mefE+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaTEM+poxtA*	*napr*
40	TET+STR+KAN+GEM+VAN+LZD	*tetL+tetO+tetS+ermB+mefE+aphA-1+aphA-3+aadA1/aadA2+cat2+blaTEM+poxtA*	*napr*
41	TET+STR+KAN+GEM+CLI+CHL+ERY+SSS+LEV+VAN+CTX+FEP	*tetL+tetO+tetS+ermB+mefE+aphA-1+aphA-3+aadA1/aadA2+cat1+cat2+blaTEM+poxtA*	*napr+bca*
42	TET+STR+KAN+GEM+CLI+CHL+ERY+VAN+CTX	*tetL+tetO+aphA-1+aadA1/aadA2+cat1+blaTEM+vanA*	*napr*
43	TET+STR+KAN+GEM+CLI+CTX	*aadA1/aadA2+poxtA*	*napr*

44	TET+STR+KAN+GEM+ERY	*ermB+aphA-1+aadA1/aadA2+cat2+blaTEM*	*napr*
45	TET+STR+K+GEM+CLI+VAN	*ermB+aphA-1+t1+cat2+blaTEM+vanA+vanB*	*napr*
46	TET+STR+KAN+GEM+CTX	*tetO+aphA-1+aadA1/aadA2cat1+blaTEM*	*napr*
47	TET+STR+KAN+GEM+ERY+CTX	*tetL+tetM+ermB+aphA-1+at2+blaTEM+vanA*	*napr*
48	TET+STR+KAN+GEM+CLI+ERY+SSS	*aphA-1+adA1/aadA2+cat1+cat2+blaTEM+vanB*	*napr+bca*
49	TET+STR+KAN+GEM+CLI+SSS+LEV+CTX	*ermB+aphA-1+cat1+cat2+blaTEM+poxtA*	*napr+bca*
50	TET+STR+KAN+GEM+CTX	*ermB+aphA-1+aadA1/aadA2+cat1+cat2+blaTEM*	*napr*
51	TET+STR+KAN+GEM+CHL+ERY+VAN+LZD+CTX+FEP	*ermB+aphA-1+aadA1/aadA2+cat1+cat2+blaTEM+poxtA*	*napr*
52	TET+STR+KAN+GEM+CLI+CHL+VAN	*ermB+aphA-1+blaTEM*	*napr*
53	TET+STR+KAN+GEM+CLI+CHL+LEV	*ermB+aphA-1+blaTEM*	*napr*
54	TET+STR+KAN+GEM+CHL+ERY+VAN+LZD+CTX	*ermB+aphA-1+blaTEM*	*napr*
55	TET+STR+KAN+GEM+VAN+CTX	*ermB+aphA-1+blaTEM*	*napr*
56	TET+STR+KAN+GEM+VAN+CTX	*tetL+ermB+aphA-1+blaTEM+poxtA*	*napr*
57	TET+STR+KAN+GEM+CLI+ERY+SSS+LEV	*ermB+aphA-1+blaTEM*	*napr*
58	TET+STR+KAN+GEM+CLI+SSS+VAN+LZD+CTX+FEP	*ermB+aphA-1+aadA1/aadA2+cat1*	*napr*
59	TET+STR+KAN+GEM	*aphA-1*	*napr*
60	TET+STR+KAN+GEM+CTX	*tetL+ermB+aphA-1+aad-6+blaTEM*	*napr*

Abbreviations: TET, tetracycline; STR, streptomycin; KAN, kanamycin; GEM, gentamycin; CLI, clindamycin; CHL, chloramphenicol; ERY, erythromycin; SSS, sulphamethoxazole; LEV, levofloxacin; VAN, vancomycin; LZD, linezolid; CTX, cefotaxime; FEP, cefepime.

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study carried the *napr* gene, which is higher than Kaczorek's finding, but only 16.67% have the *eno* gene, which is lower than their study.[34] Hence, the *napr* gene encoding nephritis-associated plasminogen-binding receptor may be the main reason for *S. dysgalactiae* infection in dairy cows.[35] Lamining-binding protein encoded by the *lmb* gene plays a key part in promoting adhesion to host laminin, which has been detected in *S. dysgalactiae* isolated from human sources.[36] Consistent with the literature, we just found two strains harbored *lmb*. The β-hemolysin encoded by the *cyl* gene is mainly involved in enhancing bacteria invasion of the host.[37] Our result shows that 19.23% of strains have this gene, which is higher than the previous report.[38] In addition, we also tested gene *bca* and *bac* severally encoding C alfa protein and C beta protein, a kind of antigen as a surface protein promoting the bacteria to enter the host cells.[39,40] In this study, 6.67% of the isolates present the *bca* gene, but none had a *bac* gene, similar to the previous study.[38] However, in contrast to earlier findings, Tian et al discovered both genes in *streptococci*.[14] The *scpB* gene can code the surface enzyme ScpB, a C5a peptidase, which can hinder the aggregation of neutrophils and combine with fibronectin to promote bacterial invasion of epithelial cells.[41] The expression of the *scpB* gene is one of the main determinants of severe neonatal infection caused by *Streptococcus B*.[42] In our study, 20% of *S. dysgalactiae* carried the *scpB* gene, which may be an important reason for causing clinical mastitis in dairy cows, and further studies are needed to confirm it. CAMP factors are encoded by the *cfb* gene, which can form pores in the host-cell membrane, mainly in *S. agalactiae*.[43] We discovered 36.67% of *S. dysgalactiae* isolates had the *cfb* gene against a previous report that only found it in *S. agalactiae*.[38] *S. agalactiae* can transfer its genetic material to *S. dysgalactiae* to adapt to the bovine environment.[44] And other studies also prove that virulence factors can be transferred between bacteria through the food chain, posing a serious threat to public health.[45,46] It may lead to complex virulence factors in *Streptococcus*, which could make controlling, treatment and prevention of bovine mastitis difficult in the future.

Conclusion

In conclusion, this study reveals that the prevalence of *S. dysgalactiae* is not high but with a high level of resistance to frequently used antibiotics across the surveyed dairy farms. Therefore, it is suggested that susceptibility test should be used as a part of diagnosis to guide selecting the appropriate drugs. And in this area, the *napr* gene is the major invasive factor in *S. dysgalactiae* to cause clinical mastitis, which largely affects the course and treatment of bovine mastitis. These findings provide a reference for public health security and convey a better understanding of the bacteria's main virulence mechanisms for further study, contributing to the development of targeted prevention programs and the establishment of clinic treatment programs in this region.

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Disclosure

The authors report no conflict of interests related to this work.

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