Infection and Drug Resistance

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ORIGINAL RESEARCH

Prevalence and Characterization of *Serratia marcescens* Isolated from Clinical Bovine Mastitis Cases in Ningxia Hui Autonomous Region of China

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Purpose: This study aimed to investigate the prevalence and genetic characterization of *Serratia marcescens* isolates from clinical bovine mastitis in Ningxia Hui Autonomous Region of China.

Methods: *S. marcescens* was identified by the polymerase-chain reaction of 16S rRNA gene and sequencing. Antimicrobial susceptibility was tested by the disk diffusion method. Genes of resistance and virulence were determined by the PCR.

Results: Overall, *S. marcescens* were confirmed from 32 of 2897 (1.1%) mastitis milk samples. These isolates showed high resistance to cefazolin (30/32, 93.8%) and chloramphenicol (28/32, 87.5%). A 12.5% (4/32) of the isolates displayed multidrug resistance (MDR). The most prevalent resistant genes found in *S. marcescens* were *TEM* (32/32, 100%) and *CTX-M* (24/32, 75.0%; *CTX-M-15*, 14/32, 43.8%; *CTX-M-14*, 8/32, 25.0%; *CTX-M-65*, 2/32, 6.3%) for extended-spectrum beta-lactamase, *cmlA* (28/32, 87.5%) and *floR* (16/32, 50.0%) for chloramphenicol resistance, *SIM-1* (2/32, 6.3%) for carbapenemases, and *sdeB* (28/32, 87.5%), *sdeY* (26/32, 81.3%), *sdeR* (26/32, 81.3%) and *sdeD* (20/32, 62.5%) for efflux pumps. Moreover, all isolates carried virulence genes *flhD, entB*, and *kpn*, and most of them contained *mrkD* (30/32, 93.8%), *ycfM* (26/32, 81.3%), *bsmB* (26/32, 81.3%), *pigP* (26/32, 81.3%), *kfu* (24/32, 75.0%) and *shlB* (24/32, 75.0%).

Conclusion: To our knowledge, this is the first report of genetic determinants for antimicrobial resistance and virulence in *S. marcescens* isolated from bovine mastitis cases in China. These findings are useful for developing strategies for prevention and treatment of bovine mastitis caused by *S. marcescens* in China.

Keywords: *Serratia marcescens*, bovine mastitis, antimicrobial resistance, virulence

Introduction

Bovine mastitis is one of the most prevalent and costly diseases affecting the dairy cattle industry.[1] The etiopathology of this disease is usually involved three factors: exposure to microorganisms, host defense mechanisms, and environmental conditions.[2] Although considerable progress has been made in controlling contagious mastitis pathogens through improved milking hygiene, little has been achieved against environmental pathogens, such as *Serratia marcescens*, which is considered an important opportunistic pathogen and has been found to be associated with outbreaks of mastitis in dairy cows.[3]

Antibiotic therapy is the main method of treating infections caused by *S. marcescens*.[4] However, the therapeutic effectiveness has been attenuated by emerging resistant strains.[5] Meanwhile, the common multidrug-resistant nature of *S. marcescens* complicates the treatment of its infections.[6] Indeed, lines of studies revealed an alarming increase in *S. marcescens* resistance to the commonly used beta-lactams.[7] Antimicrobial resistance of *S. marcescens* is mainly attributed to different resistance determinants, such as genes of extended-spectrum beta-lactamase (eg, *SHV, TEM*, and *CTX*) and

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carbapenemases (eg, *OXA-48, KPC*, and *NDM*) for beta-lactam resistance and bacterial effector proteins (eg, *sdeB, sdeD*, and *sdeY*) for multidrug resistance. Moreover, the pathogenicity of *S. marcescens* is mediated by an arsenal of virulence factors including hemolysin, lipase, protease, prodigiosin, and motility.[8]

At present, reports of *S. marcescens* are limited worldwide, especially isolates from bovine mastitis in China. The purpose of this study was to investigate the prevalence, antimicrobial resistance, and virulence genes of *S. marcescens* in bovine mastitis cases in Ningxia Hui Autonomous Region of China. To the best of our knowledge, this is the first report of genetic determinants for antimicrobial resistance and virulence in *S. marcescens* isolated from bovine mastitis cases in China.

Materials and Methods

Experimental Design

From July 2018 to May 2022, this study collected milk samples of clinical bovine mastitis from 19 commercial dairy herds in the Ningxia Hui Autonomous Region of China. Quickly place the collected sample in liquid nitrogen and transport it back to the key laboratory of new animal drug project of Gansu province. Store it in a refrigerator at −80°C until the strains in the sample are isolated and purified. All purified isolates were confirmed with PCR. *Serratia marcescens* was selected and tested for drug resistance to detect the type and quantity of drug resistance and virulence genes carried.

Sample Collection and *S. marcescens* Isolation

Mastitis milk samples of 2897 cows were collected from 19 commercial dairy herds in Ningxia Hui Autonomous Region of China during July 2018 to May 2022. In this study, clinical examinations were conducted on the breasts and nipples of lactating cows according to Jonathan et al.[9] In short, check the symmetry of one quarter of each cow's breasts. Then, possible fibrosis, inflammation and swelling, visible damage, tick infestation, tissue atrophy, and swelling of lymph nodes on the breast are detected through palpation. Test the viscosity and appearance of milk secretion from each breast segment for the presence of clots, thin sections, blood, and water secretions, in order to determine clinical mastitis.[10] After delivery to the laboratory, a volume of 20 μL of each mastitis sample was cultivated on agar plates supplemented with 5% defibrinated sheep blood (Huan kai, Guangdong, China) at 37 °C aerobically for 18 to 24 h. Presumptive *S. marcescens* colonies based on morphology and gram strain were further identified by PCR and sequencing as described in our previous study.[1] Briefly, the genomic DNA was extracted using the Bacterial DNA Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The 16S rRNA gene was amplified by the 16S rDNA Bacterial Identification PCR Kit (Takara, Shiga, Japan) according to the manufacturer's recommendations (https://www.takarabiomed.com.cn/DownLoad/RR176.pdf). The PCR products were purified and sequenced by Sanger sequencing by Sangon Biotech (Shanghai) Co., Ltd. in China. The nucleotide sequences obtained were analyzed with the NCBI-BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of *S. marcescens* to 15 antimicrobial agents was determined by disk diffusion method on Mueller–Hinton agar (MHA; Oxoid, United Kingdom) according to the Clinical and Laboratory Standards Institute (CLSI, 2018). Antimicrobial agents tested in this study include ampicillin (10 μg), cefazolin (30 μg), cefepime (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), ertapenem (10 μg), meropenem (10 μg), imipenem (10 μg), gentamicin (10 μg), tetracycline (30 μg), levofloxacin (5 μg), chloramphenicol (30 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains. Multidrug resistance was defined as isolates that were resistant to at least 3 classes of the tested antimicrobial agents.[11]

Genetic Determinants for Antimicrobial Resistance and Virulence

Genes encoding extended-spectrum beta-lactamase (*SHV, TEM, CTX-M*),[12] carbapenemases (*IMP, SPM, AIM, VIM, OXA-48, GIM, BIC, SIM-1, NDM, DIM, KPC, GES*, and *IMI*),[13,14] chloramphenicol resistance (*cmlA* and *floR*),[15] and efflux pumps (*sdeB, sdeD, sdeR, sdeS*, s*deY*, and *hasF*) were detected by PCR as previously described.[16]

Meanwhile, virulence genes for movement (*fimA* and *fimC*: fimbriae; *flhD*: flagella), adhesins (*fimH*: viscous protein expressed in type 1 fimbriae; *kpn*: fimbriae adhesins; *mrkD*: type 3 fimbria adhesin subunit; *rmpA*: regulator of the mucoid phenotype; *magA*: mucoviscosity-associated gene; *bsmB*: genes for protease and lipase production; *ycfM*: outer membrane lipoprotein), allantoin metabolism-associated gene (*allS*), siderophores (*iutA*: iron uptake transport; *entB*: enterobactin; *fyuA, ybtS, irp-1*, and *irp-2*: yersiniabactin; *kfu* and *iroN*), protectines or invasins (*K2*: type 2 capsular polysaccharide; *traT*: surface exclusion protein), and toxins (*pigP, pigA* and *pigC*: prodigiosin; *shlA, shlB*, and *hlyA*: hemolysins; *phlA*: phospholipase) were also determined though PCR.[17–22] The PCR products were analyzed by electrophoresis on 1.2% agarose gel and stained with gel-red (Tsingke, Xi'an, China). The results were visualized and photographed by a UV transilluminator. Additionally, DNA sequencing was carried out on genes of extended-spectrum beta-lactamase and carbapenemases. The sequence analysis of these genes was similar to that of the 16S rRNA gene.

Results

Bacterial Isolates

Overall, 32 (32/2897, 1.1%) *S. marcescens* isolates were obtained from 2897 mastitis milk samples from Ningxia Hui Autonomous Region of China. *S. marcescens* isolates showed resistance to cefazolin (30/32, 93.8%), chloramphenicol (28/32, 87.5%), ampicillin (12/32, 37.5%), gentamicin (4/32, 12.5%), trimethoprim/sulphamethoxazole (4/32, 12.5%), tetracycline (4/32, 12.5%), cefotaxime (2/32, 6.3%), and meropenem (2/32, 6.3%). However, all tested isolates were susceptible to ceftazidime, ertapenem, levofloxacin, cefepime, and imipenem. Besides, 4 (4/32, 12.5%) of *S. marcescens* isolates displayed multidrug resistance.

Genotypic Resistance Profiles of *S. marcescens*

The results of resistant genes showed that all *S. marcescens* isolates carried *TEM* (32/32, 100%). And most of them contained *cmlA* (28/32, 87.5%), *sdeB* (28/32, 87.5%), *sdeY* (26/32, 81.3%), *sdeR* (26/32, 81.3%), *sdeD* (20/32, 62.5%), *CTX-M* (24/32, 75.0%; *CTX-M-15*, 14/32, 43.8%; *CTX-M-14*,8/32, 25.0%; *CTX-M-65*, 2/32, 6.3%) and *floR* (16/32, 50.0%). A few isolates harbored *hasF* (10/32, 31.3%), and *SIM-1* (2/32, 6.3%). While genes encoding for extended-spectrum beta-lactamase (*SHV*), carbapenemases (*IMP, SPM, AIM, VIM, OXA-48, GIM, BIC, NDM, DIM, KPC, GES*, and *IMI*) and efflux pumps (*sdeS*) were not detected in any of the isolates (Table 1 and Supplementary Figure 1–24).

Genotypic Virulence Profiles of *S. marcescens*

We also detected the virulence-encoding genes of the *S. marcescens* isolates (Table 1). All *S. marcescens* isolates carried *flhD, kpn*, and *entB*, followed by *mrkD* (30/32, 93.8%), *bsmB* (26/32, 81.3%), *ycfM* (26/32, 81.3%), *pigP* (26/32, 81.3%), *kfu* (24/32, 75.0%), *shlB* (24/32, 75.0%), *allS* (22/32, 68.8%), *phlA* (18/32, 56.3%) *fimA* (16/32, 50.0%), *fimC* (16/32, 50.0%), *fimH* (16/32, 50.0%), *irp-2* (12/32, 37.5%), *traT* (12/32, 37.5%), *pigC* (12/32, 37.5%), *ybtS* (16/32, 50.0%), *fyuA* (8/32, 25.0%), *shlA* (8/32, 25.0%), *hlyA* (2/32, 6.3%), and *iutA* (2/32, 6.3%). Notably, each of the tested isolates carried at least 8 virulent genes. In addition, all isolates were negative for *rmpA, magA, irp-1, iroN, K2*, and *pigA* (Table 1 and Supplementary Figure 25–52).

Discussion

S. marcescens is an Enterobacteriaceae microorganism that is widespread in the environment,[23] which can cause both clinical and subclinical mastitis outbreaks during the lactation and the dry period in dairy cows.[24,25] In this study, *S. marcescens* was identified in 1.10% of the mastitis milk samples. The incidence was similar to a previous study reported in China that 1.5% of the bovine mastitis samples were positive for *S. marcescens* in bovine mastitis,[26] but lower than the 4.5% reported in Korea and 35–39% in outbreaks of mastitis in Finland.[3] These frequency variations may be due to different sample sizes, different seasons, or geographical discrepancies.[27]

S. marcescens is an emerging pathogen with increasing clinical importance due to its intrinsic resistance to different antimicrobials.[28] In this study, *S. marcescens* isolates showed high resistance to cefazolin and chloramphenicol. Similar

Table 1 Resistance Determinants and Virulence Genes of *Serratia marcescens* Isolates from Clinical Bovine Mastitis

Isolates	Resistance Pattern[a]	Resistance Genes[b]	Virulence Genes[c]
1	CFZ, CHL	*TEM, CTX-M, cmlA, floR, sdeR, sdeY*	*fimA, fimC, flhD, fimH, kpn, mrkD, bsmB, ycfM, entB, fyuA, ybtS, traT, pigC, pigP, shlB*
2	CFZ, CHL	*TEM, CTX-M, cmlA, floR, sdeB, sdeD, sdeR, sdeY, hasF*	*fimA, fimC, flhD, fimH, kpn, mrkD, bsmB, ycfM, allS, entB, iutA, ybtS, kfu, pigP, shlA, shlB, phlA*
3	AMP, CFZ, GEN, TET, CHL, COT	*TEM, CTX-M, cmlA, floR, sdeY, sdeB, sdeD, sdeR, hasF*	*fimA, fimC, flhD, kpn, mrkD, ycfM, allS, entB, ybtS, pigP, phlA*
4	AMP, CFZ, CHL	*TEM, cmlA, sdeB, sdeR, sdeY*	*flhD, kpn, mrkD, allS, entB, ybtS, pigP, shlA, shlB, phlA*
5	AMP, CFZ, GEN, TET, CHL, COT	*TEM, cmlA, floR, sdeB, sdeD*	*fimA, flhD, kpn, bsmB, allS, entB, kfu*
6	CFZ, CHL	*TEM, cmlA, sdeY, sdeB, sdeR*	*flhD, fimH, kpn, mrkD, bsmB, allS, entB, kfu, traT, pigP, shlB*
7	CTX, CHL	*TEM, CTX-M, cmlA, sdeY, sdeB, sdeD, sdeR*	*fimA, fimC, flhD, kpn, mrkD, bsmB, ycfM, allS, entB, kfu, pigC, pigP, shlA, shlB, phlA*
8	AMP, CFZ, CHL	*TEM, CTX-M, cmlA, sdeB, sdeD, sdeR, sdeY*	*fimA, fimC, flhD, kpn, mrkD, bsmB, ycfM, allS, entB, kfu, pigC, pigP, shlA, shlB, phlA*
9	CFZ, CHL	*TEM, CTX-M, cmlA, floR, sdeB, sdeD, sdeR, sdeY*	*flhD, fimH, kpn, mrkD, pigP, ycfM, entB, fyuA, irp-2, kfu, traT, shlB, phlA*
10	CFZ, CHL	*TEM, CTX-M, cmlA, floR, sdeB, sdeD, sdeR, sdeY*	*flhD, fimH, kpn, mrkD, bsmB, ycfM, allS, entB, irp-2, fyuA, kfu, traT, pigP, shlB, phlA*
11	CFZ, MEM, CHL	*TEM, CTX-M, SIM-1, cmlA, floR*	*flhD, fimH, kpn, mrkD, bsmB, ycfM, entB, kfu, traT*
12	AMP, CFZ, CHL	*TEM, CTX-M, cmlA, floR, sdeB, sdeD, hasF*	*fimA, flhD, kpn, mrkD, bsmB, ycfM, allS, entB, irp-2, kfu*
13	CFZ, CHL	*TEM, cmlA, sdeB, sdeR, sdeY, hasF*	*fimA, fimC, flhD, kpn, mrkD, bsmB, ycfM, allS, entB, ybtS, irp-2, kfu, pigC, pigP, shlB, phlA*
14	CFZ	*TEM, CTX-M, sdeB, sdeD, sdeR, sdeY*	*flhD, kpn, mrkD, bsmB, ycfM, allS, entB, ybtS, irp-2, kfu, pigP, shlB*
15	CFZ, CHL	*TEM, CTX-M, cmlA, sdeB, sdeR, sdeY*	*fimC, flhD, fimH, kpn, mrkD, bsmB, ycfM, entB, ybtS, pigC, pigP, shlB*
16	AMP, CFZ	*TEM, CTX-M, sdeB, sdeD, sdeR, sdeY, hasF*	*fimC, flhD, fimH, kpn, mrkD, bsmB, ycfM, fyuA, entB, ybtS, irp-2, kfu, traT, pigC, pigP, shlB, hlyA, phlA*
17	AMP, CFZ, CHL	*TEM, CTX-M, cmlA, sdeB, sdeD, hasF*	*fimA, flhD, kpn, mrkD, bsmB, ycfM, allS, entB, irp-2, kfu*
18	CFZ, CHL	*TEM, CTX-M, cmlA, sdeR, sdeY*	*fimA, fimC, flhD, fimH, kpn, mrkD, bsmB, ycfM, entB, fyuA, ybtS, traT, pigC, pigP, shlB*
19	AMP, CFZ, CHL	*TEM, CTX-M, cmlA, floR, sdeB, sdeD, sdeR, sdeY*	*fimA, fimC, flhD, kpn, mrkD, bsmB, ycfM, allS, entB, kfu, pigC, pigP, shlB, phlA*
20	AMP, CFZ, GEN, TET, CHL, COT	*TEM, CTX-M, cmlA, floR, sdeB, sdeD, sdeR, sdeY, hasF*	*fimA, fimC, flhD, kpn, mrkD, ycfM, allS, entB, ybtS, pigP, phlA*
21	CFZ	*TEM, CTX-M, sdeY, sdeB, sdeD, sdeR*	*flhD, kpn, mrkD, bsmB, ycfM, allS, entB, ybtS, irp-2, kfu, pigP, shlA, shlB*
22	CFZ, CHL	*TEM, CTX-M, cmlA, floR, sdeB, sdeD, sdeR, sdeY, hasF*	*fimA, fimC, flhD, fimH, kpn, mrkD, bsmB, ycfM, allS, iutA, entB, ybtS, kfu, pigP, shlA, shlB, phlA*
23	CFZ, CHL	*TEM, CTX-M, cmlA, sdeB, sdeD, sdeR, sdeY*	*flhD, fimH, kpn, mrkD, ycfM, entB, irp-2, fyuA, kfu, traT, pigP, shlB, phlA*
24	CFZ, MEM, CHL	*TEM, CTX-M, SIM-1, cmlA, floR*	*flhD, fimH, kpn, mrkD, bsmB, ycfM, entB, kfu, traT*
25	CFZ, CHL	*TEM, cmlA, sdeB, sdeR, sdeY, hasF*	*fimA, fimC, flhD, kpn, mrkD, bsmB, ycfM, allS, entB, ybtS, irp-2, kfu, pigC, pigP, shlB, phlA*
26	CFZ, CHL	*TEM, cmlA, sdeB, sdeR, sdeY*	*flhD, fimH, kpn, mrkD, bsmB, allS, entB, kfu, traT, pigP, shlB*
27	AMP, CFZ	*TEM, CTX-M, sdeB, sdeD, sdeR, sdeY, hasF*	*fimC, flhD, fimH, kpn, mrkD, bsmB, ycfM, entB, irp-2, fyuA, ybtS, kfu, traT, pigC, pigP, shlB, hlyA, phlA*
28	AMP, CFZ, GEN, TET, CHL, COT	*TEM, cmlA, floR, sdeB, sdeD*	*fimA, flhD, kpn, bsmB, allS, entB, kfu*
29	CFZ, CHL	*TEM, CTX-M, cmlA, floR, sdeB, sdeR, sdeY*	*fimC, flhD, fimH, kpn, mrkD, bsmB, ycfM, allS, entB, ybtS, pigC, pigP, shlA, shlB*
30	AMP, CFZ, CHL	*TEM, cmlA, floR, sdeB, sdeR, sdeY*	*flhD, kpn, mrkD, entB, ybtS, pigP, shlB, phlA*
31	CTX, CHL	*TEM, CTX-M, cmlA, sdeB, sdeD, sdeR, sdeY*	*fimA, fimC, flhD, kpn, mrkD, bsmB, ycfM, allS, entB, kfu, pigC, pigP, shlA, shlB, phlA*
32	CFZ, CHL	*TEM, CTX-M, cmlA, floR, sdeB, sdeD, sdeR, sdeY*	*flhD, fimH, kpn, mrkD, bsmB, ycfM, allS, entB, irp-2, fyuA, kfu, traT, pigP, shlB, phlA*

Abbreviations: [a]AMP, ampicillin, CFZ, cefazolin, CTX, cefotaxime, MEM, meropenem, GEN, gentamicin, TET, tetracycline, CHL, chloramphenicol, COT, trimethoprim/sulfamethoxazole; [b]*TEM, CTX-M*, Genes encoding extended-spectrum beta-lactamase; *SIM-1*, carbapenemases; *cmlA* and *floR*, chloramphenicol resistance; *sdeB, sdeD, sdeR, sdeY*, and *hasF*, efflux pumps; [c]*fimA* and *fimC*, fimbriae; *flhD*, flagella; *fimH*, viscous protein expressed in type 1 fimbriae; *kpn*, fimbriae adhesins; *mrkD*, type 3 fimbria adhesin subunit; *bsmB*, genes for protease and lipase production; *ycfM*, outer membrane lipoprotein; *allS*, allantoin metabolism-associated gene; *iutA*, iron uptake transport; *entB*, enterobactin; *fyuA, ybtS, irp*-2, yersiniabactin; *kfu*, siderophores; *traT*, surface exclusion protein; *pigP*, and *pigC*, prodigiosin; *shlA, shlB*, and *hlyA*, hemolysins; *phlA*, phospholipase.

results were reported that *S. marcescens* and other Enterobacteriaceae isolates from bovine mastitis frequently exhibited resistance to these antimicrobials.[1,29,30] We found that *Serratia marcescens* is resistant to cefazolin and sensitive to Cefepime, Ceftazidime, Cefotaxime, etc. This result is the same as that of previous studies.[31] Cefazolin and chloramphenicol are broad-spectrum antibiotics with therapeutic effects on bovine pathogens.[32] The high resistance could be due to the long-term and widespread use of these antimicrobials on dairy farms.[33] In addition, an increase in exposure to antimicrobial drugs can lead to an increase in drug-resistant strains.[34] Veterinarians on farms may be exposed to chloramphenicol environments, and the chloramphenicol-resistant bacteria they carry are then transferred to Serratia marcescens through plasmid mediated transfer. Carbapenems are the last resort β-lactam antibiotics for treating infections caused by multidrug-resistant Gram-negative bacteria. Unfortunately, carbapenem-resistant pathogens have emerged with the increasing clinical use of carbapenems and now pose a great threat to human health.[35] In our study, although only 2 *S. marcescens* isolates exhibited carbapenem resistance, their potential threat should not be ignored.

The phenotypic resistance is mainly conferred by corresponding genes, which can be transferred to other bacteria and pose a serious threat to public health through the food chain.[36] The most common antimicrobial resistance found in our study was to cefazolin and chloramphenicol. Thus, the resistant genes against β-lactams and chloramphenicol were detected. We found that all *S. marcescens* isolates carried *TEM* alone or combined with *CTX-M* and showed resistance to at least one of the tested β-lactams. Similarly, the *cmlA* was found in all chloramphenicol-resistant isolates alone or in combination with *floR*. These results indicating that genes *TEM* and *cmlA* may play an important role in β-lactams and chloramphenicol resistance in *S. marcescens* isolates from bovine mastitis in Ningxia Hui Autonomous Region of China, respectively. These findings were consistent with other reports that these genes were frequently observed in Enterobacteriaceae isolates from food producing animal in China and other countries.[30,37,38] Carbapenems have been the most successful β-lactams in evading bacterial resistance. However, carbapenem resistance, mediated by acquired carbapenemase genes, has been increasingly reported.[39] It is noteworthy that we found the two carbapenem-resistant *S. marcescens* isolates were positive for the carbapenemase gene *SIM-1*. To the best of our knowledge, this is the first report of a *SIM-1* producing *S. marcescens* from bovine mastitis in China. Additionally, efflux pumps also play important roles in β-lactams and chloramphenicol resistance, as well as the multidrug resistance.[40] Past studies reported that *S. marcescens* enhanced its infectivity and its inherent resistance to antibiotics through plasmids and efflux pumps, which greatly contributed to the emergence of multiple drug-resistant strains and finally complicated the treatment process,[41,42] and the underlying mechanisms were complicated.[43] In our study, regardless of the two *S. marcescens* isolates resistant to cefazolin, meropenem, and chloramphenicol simultaneously, the efflux pump-related genes *sdeB, sdeD, sdeR, sdeS*, s*deY*, or *hasF* (in combination) were observed in all β-lactams and chloramphenicol-resistant isolates, respectively. Efflux pump is helpful for bacteria to excrete antibiotics, and it is the main cause of clinical intrinsic and acquired multidrug resistance in bacteria.[44] Our results showed that only 12.5% of the *S. marcescens* isolates were multidrug resistance. This disparity could be due to the limited antimicrobial classes tested in our study.

Pathogenic microorganisms normally express several virulence determinants facilitating their invasion and evasion of the host defenses and cause disease.[45] Bacteria adhere to the tissues employing fimbria or pili, thereby establishing infection.[46] Fimbriae of *S. marcescens* encoded by *fimA, fimC* and *fimH* play vital roles in surface attachment and colonization.[47] Additionally, flagella-controlled swarming motility is governed by *flhD* and contributes to cell surface attachment.[18,48] In this study, all *S. marcescens* carried *flhD* gene, and 50.0% of the isolates were positive for *fimA* and *fimC*. Besides, we also found that all *S. marcescens* isolates harbored the adhesions protein-related virulence gene *kpn*, the majority of the isolates carried *mrkD* (93.8%), *ycfM* (81.3%) and *bsmB* (81.3%). The *kpn* gene encodes *fimH*-like pilus adhesin, and the *ycfM* gene encodes outer membrane lipoproteins. Both of them play an important role in adhesion and colony formation.[49] The *bsmB* gene plays a key role in virulence factor production such as protease, serralysin, lipase and S-layer protein synthesis.[50] Moreover, previous study confirmed that nitrogen sources in environment may affect the adhesion of *S. marcescens*.[51] In our study, 68.8% of the *S. marcescens* isolates were positive for the allantoin metabolism-related gene *allS*, which helps pathogens compete for nitrogen sources with other bacteria.[52] The high prevalence of the adhesion-related factors mentioned above in our study may provide strong abilities to attach both living tissues and non-living objects, a feature that enables *S. marcescens* to colonize within host tissues and participate in the first stage of infections.

Iron carriers are critical virulence determinants involved in bacterial pathogenesis, invasiveness, and molecular competition at the host pathogen interface.[53] Meanwhile, the carriers enable pathogens to overcome the isolation of iron ions by the immune system of eukaryotic cells and capture metals.[53] In this study, all *S. marcescens* isolates carried the metal-chelating siderophore enterobactin encoding gene *entB*. The phenolate-type siderophore yersiniabactin encoding genes *ybtS* (50.0%), *irp-2* (37.5%), and *fyuA* (25.0%) were frequently observed in the tested isolates alone or in combination. Meanwhile, the iron-uptake system-related genes *iutA* and *kfu* were determined in 6.3% and 75.0% of the *S. marcescens* isolates, respectively. The *iutA* gene encoding the ferric aerobactin receptor involves typical ATP binding cassette (ABC)-type transporters.[54] The ferric uptake transporter encoded by *kfu* mainly mediates the uptake of ferric iron.[19] Previous study found that the highly virulent strains were more likely to carry the ferric ion carriers.[19] High frequency of the genes associated with siderophore activation and the iron-uptake system imply that the *S. marcescens* isolates tested in this study may have a strong ability to capture ions, which is contributive to invade the mammary epithelial cells and induce bovine mastitis.

The *traT* gene, encoding a surface exclusion protein, is the most common determinant of virulence factors, and generally considered to be related to serum drug resistance.[55,56] In this study, 37.5% of *S. marcescens* isolates carried the *traT* gene. To our knowledge, this gene was rarely reported in bovine *S. marcescens*. Prodigiosin is a prominent red pigment produced by *S. marcescens* and is essential for invasion, survival, and pathogenicity.[18,57] We tested the prodigiosin biosynthesis-related genes and found the most frequent gene was *pigP* (81.3%), followed by *pigC* (37.5%). *PigP* is a positive regulator of prodigiosin production that regulates swarming and hemolysis through serratamolide production.[16] Other studies also reported that the *pigP* was more prevalent in *S. marcescens* isolates than *pigA* and *pigC*.[16,58]

Hemolysin is a kind of exotoxin, which has hemolytic and cytotoxic activities and plays an important role in the occurrence or aggravation of mastitis.[59,60] The hemolysin is encoded by *shlA* in *S. marcescens*. The outer membrane protein encoding gene *shlB* gene is responsible for secretion and activation of *shlA*.[61] *shlA*/*shlB* system can secrete hemolysin under low iron conditions, and may make *S. marcescens* to use hemoglobin released by hemolysis as iron source.[62] Therefore, the significant increase in hemolytic activity of hemolysin may transform the opportunistic pathogenic *S. marcescens* strain into a hypervirulent type.[63] It is noteworthy that 25.0% and 75.0% of the tested isolates in this study carried both *shlA* and *shlB*, which may be potentially hypervirulent strains. In addition, the *phlA* gene is also considered to be related to hemolysis and cytotoxicity. *phlA* itself does not directly induce membrane instability of target cells. Phospholipase A encoded by *phlA* can catalyze the production of phospholipids, which lead to hemolysis and cell death.[64] In this study, *S. marcescens* isolates also evaluated for the presence of toxin genes *phlA*. This is similar to the observed results of *S. marcescens* isolates in previous studies.[17]

Conclusion

In conclusion, although the prevalence of *S. marcescens* is low in our study, the high frequencies of phenotypic and genotypic resistance to cefazolin and chloramphenicol as well as the multidrug resistance remind the government to pay special attention to the antimicrobial agents used in dairy industry. Importantly, the emergence of carbapenem-resistant *S. marcescens* pose an alarming threat to public health due to the transmission of resistant determinants through the food chain. Furthermore, the high incidence of virulence genes *mrkD, ycfM, pigP, bsmB, flhD*, and *phlA* detected in *S. marcescens* suggested their pathogenic potential in bovine mastitis. Further investigations to be conducted to understand the pathogenicity of the individual virulent factor.

Ethics Approval and Informed Consent

The animal owners were informed about the purpose of the study and consent of each animal owner was obtained before the physical examination of cows for clinical mastitis and the collection of milk samples. All procedures involved in animal care and their use were in strict accordance with the guidelines for the Care and Use of Laboratory Animals, Lanzhou Institute of Husbandry and Pharmaceutical Sciences, CAAS, China (SYXK-2019-0012).

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Disclosure

The authors report no conflicts of interest in this work.

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