Prevalence and characterization of *Staphylococcus aureus* isolates from subclinical bovine mastitis in southern Xinjiang, China

Qiang Ren,¹ Guanghua Liao,¹ Zihao Wu,² Junfan Lv,² and Wei Chen^{1,2*}

¹Key Laboratory of Protection and Utilization of Biological Resources in Tarim Basin of Xinjiang Production and Construction Corps, College of Life Sciences, Tarim University, Alar 86-843300, China

²College of Animal Sciences, Key Laboratory of Tarim Animal Husbandry and Science Technology of Xinjiang Production and Construction Corps, Tarim University, Alar 86-843300, China

ABSTRACT

Staphylococcus aureus is one of the major pathogens causing mastitis in dairy herds. The colonization of dairy cows and subsequent contamination of raw milk by *S. aureus*, especially strains exhibiting multidrug resistance and biofilm-forming and toxin-producing abilities, remains an important issue for both dairy farmers and public health. In this study, we investigated the prevalence, antimicrobial susceptibility, biofilm formation, and genetic diversity of *S. aureus* from subclinical bovine mastitis in dairy farms located in southern Xinjiang, China. Sixty-five isolates from 84 subclinical mastitic milk samples were identified as *S. aureus*. The resistance rates to penicillin, erythromycin, clindamycin, tetracycline, gentamicin, linezolid, rifampicin, quinupudin-dafupudin, ciprofloxacin, norfloxacin, and chloramphenicol were 58.5, 44.6, 40.0, 18.5, 12.3, 10.8, 9.2, 6.2, 4.6, 4.6, and 1.5%, respectively. All isolates were susceptible to cefoxitin, sulfamethoxazole-trimethoprim, and vancomycin. Isolates from farm A showed a significantly higher resistance rate to tetracycline (16.9%) than those from farm B (1.5%). The most frequently detected virulence factors were *hla* (96.9%, 63/65) and *hly* (100.0%, 65/65). The percentage rates of the staphylococcal enterotoxin genes *sea*, *sec*, *sed*, *seg*, *seh*, *sei*, and *sej* in *S. aureus* isolates were 4.6, 33.8, 27.7, 3.1, 41.5, 41.5, and 7.7%, respectively. The percentage rate of the *sec* gene in isolates from farm B (30.8%) was significantly higher than that of farm A (3.1%). The percentage rates of the *tsst* and *pvl* genes in *S. aureus* isolates were 26.2 and 40.0%. The percentage rate of the *pvl* gene in isolates from farm B (32.3%) was significantly higher than that of farm A (7.7%). The adhesion molecules *fnaA*, *fnaB*, *clfA*, *clfB*, and *cna* were detected in 21 (32.3%), 23 (35.4%), 65 (100.0%), 65 (100.0%), and 65 (100.0%) isolates, respectively. The

percentage rates of the *icaA*, *sarA*, *tcaR*, *ccp*, *luxS*, and *sigB* genes in *S. aureus* isolates were 69.2, 100.0, 86.2, 95.4, 84.6, and 100.0%, respectively. The *fnaB* and *icaA* genes were more frequently detected in isolates from farm A (29.2 and 40.0%, respectively) than those from farm B (6.2 and 29.2%, respectively). The *luxS* gene was more often found in isolates from farm B (50.8%) than those from farm A (33.8%). Using the microplate method, 61.5, 26.2, and 10.8% of the isolates showed weak, moderate, and strong biofilm-forming abilities, respectively. Different clonal complex (CC) and *spa*-types were identified, including CC81, CC398, CC88, CC5405, and CC5406. Importantly, in this study we report for the first time 41 new sequence types (ST) among 44 distinct ST. These results indicated high genetic diversity of *S. aureus* involved in subclinical bovine mastitis in southern Xinjiang, China. The results also showed that *S. aureus* from subclinical bovine mastitis cases in southern Xinjiang, China, were mainly resistant to β -lactams, erythromycin, and clindamycin. Also, biofilm- and adhesion-related genes, which are increasingly known as important virulence factors in the pathogenesis of *S. aureus* infections, were detected at a high rate. This study could help identify predominant clones and provide surveillance measures to decrease or eliminate *S. aureus* contamination in raw milk of dairy cows with subclinical mastitis.

Key words: *Staphylococcus aureus*, raw milk, mastitis, antimicrobial resistance, molecular typing

INTRODUCTION

Staphylococcus aureus is one of the leading pathogens causing mastitis in dairy cows. It is reported that up to 40.0% of mastitis cases in China and other countries are caused by *S. aureus* (Kateete et al., 2013; Basanisi et al., 2017; Wang et al., 2018). Dairy cow mastitis is a global challenge, because it impairs animal health and welfare, decreases milk production, and increases health care costs. Thus the economic losses for the dairy industry are considerable (Hertl et al., 2010; Schroeder,

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*Corresponding author: 379497687@qq.com

2012; Wang et al., 2018). More importantly, contaminated raw milk at farm level may lead to subsequent public health problems, giving rise to association of *S. aureus* with food contamination (Jakobsen et al., 2011; Rola et al., 2016; Wang et al., 2018).

Various virulence genes in *S. aureus* encode a wide range of virulence factors, such as toxic shock syndrome toxin-1 (**TSST-1**), staphylococcus enterotoxins (**SE**), hemolysins (**HL**), and Panton-Valentine leukocidin (**PVL**; Wang et al., 2018). Staphylococcus enterotoxins are considered the major cause of *S. aureus* associated with food poisoning. It is reported that more than 90.0% of *S. aureus*-related food poisoning outbreaks were attributable to the classic SE encoded by the *sea* to *see* genes (Tarekne et al., 2016). The staphylococcal enterotoxins A and D (SEA and SED) are regarded as the major sources of food poisoning (Sato'o et al., 2014; Arfatahery et al., 2016). The TSST-1 toxin could cause toxic shock syndrome by attenuating the host immune response, and PVL could destroy host leukocytes and result in tissue necrosis (Wang et al., 2018).

Antibiotic therapy is an important measure for controlling bovine mastitis and human infections (Gomes and Henriques, 2016). However, an increasing number of studies report that *S. aureus* exhibits resistance to multiple classes of antimicrobial agents as a response to the selective pressures of antimicrobials (Gomes and Henriques, 2016). Additionally, biofilm formation is frequently associated with chronic and recurrent infections in animals and humans. Biofilm-associated protein (**Bap**) plays a key role in initial attachment and recruitment of *S. aureus* (Khoramian et al., 2015; Felipe et al., 2017). The *icaA* gene, found at the *ica* locus present in *S. aureus* and in *Staphylococcus epidermidis*, plays an important role in biofilm production (Aslantaş and Demir, 2016). The *icaA* gene encodes *N*-acetylglucosaminyltransferase responsible for the *N*-acetylglucosamine oligomers from UDP-*N*-acetylglucosamine (Arciola et al., 2001; Aslantaş and Demir, 2016). Also, *S. aureus* possess a diverse of adhesins that play crucial roles in the onset of infection by binding host tissues and are regarded as important virulence factors. Of these adhesins, fibronectin-binding proteins (*fnbA* and *fnbB*), clumping factors (*clfA* and *clfB*), and collagen-binding protein (*cna*) are considered important virulence factors in binding host cells, colonization, and invasion (Haveri et al., 2008; Aslantaş and Demir, 2016). Additionally, *S. aureus* have a variety of global regulators controlling the production of virulence factors. Staphylococcal accessory regulator (*sarA*) is a central regulatory element that is essential for the synthesis of polysaccharide intercellular adhesin (PIA) and biofilm development in this species. Under

ica-independent conditions, it is reported that *sigB* is critical to biofilm formation in *S. aureus* (Lauderdale et al., 2009).

Molecular epidemiology-based methods are important tools to analyze clonal relatedness and genetic diversity, and to track the dissemination of *S. aureus* infections (Wang et al., 2018). Molecular epidemiology-based methods have reportedly been widely used in animal husbandry, medical care, food safety testing, and other fields (Montanaro et al., 2016; Käppeli et al., 2019; Sun et al., 2019). Multilocus sequence typing (**MLST**) based on the profiles of alleles at 7 loci of housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL*) and *spa*-typing based on the variable X-region of the staphylococcal protein A gene (*spa*) have been used to confirm the highly clonal structure of *S. aureus* (Shopsin et al., 1999). Some research has found that particular *S. aureus* clonal lineages may be prevalent geographically, and exhibit specific antibiotic-resistant and virulence patterns (Hata et al., 2010; Wang et al., 2018). Thus, better knowledge about the genotypes of *S. aureus* isolates involved in dairy cow mastitis might lead to more effective measures to reduce the spread of infection and could be useful to track the sources of infection (Li et al., 2017).

The aims of the present study were (1) to study molecular genetic characteristics, (2) to investigate antibiotic susceptibility, (3) to determine the ability of biofilm formation and the related genes, and (4) to search for adhesin-related genes such as *cna*, *fnb*, and *clf* genes and enterotoxin genes in *S. aureus* isolates from subclinical mastitis.

MATERIALS AND METHODS

Isolation and Identification of *S. aureus* from Milk

According to National Mastitis Council (NMC) guidelines, a total of 84 mastitic milk samples were aseptically collected from cows with subclinical mastitis in 2 different farms of southern Xinjiang, China, including farm A (in the Akesu region; herd size ≥ 100 ; milking frequency, 2 times per day; $n = 44$ samples) and farm B (in Bayingol Mongolian Autonomous Prefecture; herd size ≥ 100 ; milking frequency, 2 times per day; $n = 40$ samples). Subclinical bovine mastitis cases were detected using the California Mastitis Test. For milk collection, the udders of cows with subclinical mastitis were cleaned with water and dried. Cotton balls with 75% ethanol were used to disinfect the surface of the udder. The first few streams of milk were discarded. The collected milk was kept in a sterile tube and transported to the laboratory within 4 h. Milk samples were

cultured in mannitol salt broth (MSB; Hope Bio, Qingdao, China) and then incubated aerobically at 37°C for 24 to 72 h, followed by streaking on Columbia blood agar (CBA; Hope Bio). The plates were incubated at 37°C for 24 to 72 h. Primary identification of *S. aureus* was performed by picking the characteristic colonies (large, creamy, and strongly hemolytic on CBA). All these potential *S. aureus* isolates were subcultured to be identified by routine biochemical properties (Atalla et al., 2008). Confirmation of identification was performed by 16S rRNA gene sequencing as well as by PCR analysis of the thermonuclease (*nuc*) gene specific to this species (Brakstad et al., 1992). The retrieved *S. aureus* isolates were kept at -20°C in tryptic soy broth (TSB; Becton Dickinson, Wokingham, UK), containing 20% volumes of glycerol, for further study.

Antibiotic Susceptibility Testing

A panel of 14 antimicrobial agents was used to detect the antibiotic susceptibility of all the tested isolates. Tetracycline (30 µg), penicillin G (10 U), linezolid (30 µg), chloramphenicol (30 µg), clindamycin (2 µg), ciprofloxacin (5 µg), gentamicin (10 µg), norfloxacin (10 µg), erythromycin (15 µg), sulfamethoxazole-trimethoprim (25 µg), rifampicin (5 µg), and quinupudin-dafupudin (15 µg) were tested via the agar diffusion (Kirby-Bauer) method using Mueller-Hinton agar (Oxoid, Waltham, MA) and commercially available discs (Oxoid) according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2015). Vancomycin susceptibility was tested via broth microdilution, according to the recommendations of the CLSI (CLSI, 2015). *Staphylococcus aureus* ATCC25923 and *S. aureus* ATCC29213 were used as quality-control strains.

Detection of MRSA, Virulence, and Biofilm-Related Genes

A cefoxitin (30 µg) disc was used for detecting methicillin-resistant isolates. *Staphylococcus aureus* ATCC25923 was used as a control. The *mecA* gene responsible for methicillin-resistant *S. aureus* (MRSA) was detected by PCR using primers *mecA* F (5'-AAAATCGATGGTAAAGGTTGGC-3') and *mecA* R (5'-AGTTCTGCAGTACCGGATTTGC-3'; Panahi and Saei, 2019).

The genes encoding PVL (*lukF*; Li et al., 2018), SE (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, and *sej*; Løvseth A et al., 2004), exfoliative toxin (*eta* and *etb*; Li et al., 2018), HL (*hla* and *hlb*; Li et al., 2018), TSST (*tsst*; Løvseth A et al., 2004), adhesion factor (*fnbA*, *fnbB*, *clfA*, *clfB*, and *cna*; Zmantar et al., 2008; Klein et al., 2012; Li et al., 2018), and biofilm-related genes (*bap*, *icaA*, *ccp*,

luxS, *sarA*, *sigB*, *agrC*, *tcaR*; Ciftci et al., 2009) were amplified via PCR.

Biofilm Formation Assay

Isolates' ability to form biofilm was assessed using a 96-well microtiter plate assay, as previously described (Pratt and Kolter, 1998; Xie et al., 2019). Briefly, cells were diluted 1:100 with fresh TSB broth and cultured at 37°C for 24 h without shaking. Biofilms were stained with crystal violet (Sigma-Aldrich, St. Louis, MO) and dissolved in 95% ethanol (wt/vol: 0.5%). The optical density was determined at 570 nm in an enzyme-linked immunosorbent assay reader (Bio-Rad, Hercules, CA). A strong biofilm-former, *S. epidermidis* ATCC35984 was selected as the positive control, and sterile TSB was used as negative control for the biofilm production assays (Xie et al., 2019). These biofilm assays were performed in triplicate including biological duplicates. An OD₅₇₀ value of 0.13 was applied as the cutoff point to distinguish between biofilm formers and non-biofilm formers [cutoff (OD_c) = average OD + 3 SD of negative control]. Biofilm formation was classified as strong (+++; OD₅₇₀ > 0.39), moderate (++; 0.39 > OD₅₇₀ > 0.26), weak (+; 0.26 > OD₅₇₀ > 0.13), or negative (-; OD₅₇₀ < 0.13).

Multilocus Sequence Typing (MLST)

Multilocus genotyping based on the sequencing of 7 housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL*) was performed on all 65 *S. aureus* isolates (Montanaro et al., 2016). Alleles and sequence type (ST) were assigned according to the *S. aureus* MLST database (<https://pubmlst.org/saureus/>). The ST were then clustered into clonal complexes (CC) used for the goeBURST algorithm by phyloviz (<http://goeBURST.phyloviz.net>).

Spa Typing

The isolates were subjected to PCR amplification for the polymorphic X region of the *spa* gene, using the standard primers *spa*-1113f (5'-TAAAGACGATCCTTCG-GTGAGC-3') and *spa*-1514r (5'-CAGCAGTAGTGC-CGTTTGCTT-3'); primers and protocol available on the Ridom Spa Server database (<http://www.spaserver.ridom.de/>). The purified PCR products of the *spa* gene were sequenced, and then *spa* type was deduced using this database. The BURP (Based Upon Repeat Patterns) algorithm analysis was performed using the Ridom StaphType software (Ridom GmbH, Münster, Germany) to cluster related *spa* types. If a *spa* repeat did not match any *spa* types, the sequence of this *spa*

Table 1. Prevalence of *Staphylococcus aureus* from subclinical bovine mastitis in dairy farms located in southern Xinjiang, China

Farm	No. of samples	No. of <i>S. aureus</i> isolates	No. of MRSA ¹ isolates
A	44	30	0
B	40	35	0
Total	84	65	0

¹MRSA = methicillin-resistant *S. aureus*.

was uploaded to the Ridom Spa Server database to assign a new type.

Statistical Analysis

The chi-squared test was calculated using SPSS 18.0 software (SPSS, Chicago, IL) to analyze the differences in the prevalence, antimicrobial resistance, distribution of virulence or biofilm-producing genes, and biofilm formation ability between the 2 farms. A *P*-value < 0.05 was considered statistically significant.

RESULTS

Isolation and Identification of *S. aureus*

A total of 65 *S. aureus* isolates were identified by morphological, biochemical methods and 16S rRNA gene sequencing in this study (Table 1). We obtained 30 and 35 *S. aureus* isolates from samples collected from farms A and B, respectively. No isolate showed resistance to cefoxitin and harbored the *mecA* gene (Table 1).

MLST and Spa Typing

Information on the isolation and identification of MLST and *spa* types is shown in Table 2. We identified 44 distinct ST within the 65 isolates, including 41 new ST (93.2%, 41/44). Of 44 ST, 27 ST (61.4%, 27/44) were found in farm A, 24 of which were new ST, and 18 ST (40.9%, 18/44) were found in farm B, 17 of which were novel. In both farms A and B, ST398 was the same ST. The goeBURST analysis clustered all 44 ST isolated from subclinical bovine mastitis into 5 CC in farm A, including CC81, CC398, and CC88, and into 3 CC, including CC398, CC5405, and CC5406, in farm B. Of these complexes, CC81 and CC398 were predominant in farm A, and CC5405 and CC5406 were predominant in farm B.

The *spa* typing discriminated 65 *S. aureus* isolates into 8 *spa* types, of which 5 were obtained in farm A and 3 were found in farm B. The most prevalent *spa* type was t5100 (40.0%, 12/30) in farm A. In addition, 4 other *spa* types (t034, t2970, t2592, and t1764) were

also detected in isolates from farm A. Isolates from farm B were defined by 3 *spa* types: t189, t571, and t519. The most predominant *spa* type was t189 (57.1%, 20/35) in farm B. Based on *spa* typing, isolates with identical *spa* types exhibited different ST (Table 2).

Antimicrobial Susceptibility Profiles

Data on the antimicrobial susceptibility of 65 *S. aureus* isolates are shown in Table 3. All *S. aureus* isolates were susceptible to vancomycin and cefoxitin. Various rates of resistance to penicillin (58.5%, 38/65), erythromycin (44.6%, 29/65), clindamycin (40.0%, 26/65), tetracycline (18.5%, 12/65), gentamicin (12.3%, 8/65), linezolid (10.8%, 7/65), rifampicin (9.2%, 6/65), quinupudin-dafupudin (6.2%, 4/65), norfloxacin (4.6%, 3/65), ciprofloxacin (4.6%, 3/65), and chloramphenicol (1.5%, 1/65) were detected in this study. Isolates from farm A showed significantly higher resistance to tetracycline (16.9%) than those from farm B (*P* < 0.05; Table 4 and Table 5). Of 65 *S. aureus* isolates, 27 (41.5%) strains showed a multidrug-resistant phenotype (**MDR**, resistant to 3 or more classes of antimicrobial agents; Table 3; Figure 1).

The resistance profiles of *S. aureus* isolates differed by their CC or *spa* types (Table 5). Of CC398-t034 isolates, 90% exhibited the MDR phenotype. We found that CC5406-t519 isolates displayed the highest rates of resistance to erythromycin (100.0%) and clindamycin (91.7%).

Detection of Virulence Genes

All isolates harbored at least 1 virulence gene (Table 6; Figure 1). Of the 15 investigated virulence genes (*tsst*, *pvl*, *eta*, *etb*, *hla*, *hly*, *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, and *sej*), the *hly*, *hla*, *pvl*, and *tsst* genes were detected in 65 (100.0%), 63 (96.9%), 26 (40.0%), and 17 (26.2%) isolates, respectively (Table 6; Figure 1). Of the 9 investigated enterotoxin genes, *seh*, *sei*, *sec*, *sed*, *sej*, *sea*, and *seg* were detected in 27 (41.5%), 27 (41.5%), 22 (33.8%), 18 (27.7%), 5 (7.7%), 3 (4.6%), and 2 (3.1%) isolates, respectively (Table 6; Figure 1). The detection frequency of *pvl* and *sec* showed signifi-

cant difference in isolates from farm B (32.3 and 30.8%, respectively) than those from farm A (7.7 and 3.1%, respectively; $P < 0.05$; Table 6). The enterotoxin genes *seb* and *see* were not found in any isolate.

Biofilm-Related Genes and Biofilm Formation Ability

Of the 13 investigated biofilm-related genes (*fnbA*, *fnbB*, *clfA*, *clfB*, *cna*, *icaA*, *sarA*, *bap*, *tcaR*, *ccp*, *luxS*, *agrC*, *sigB*), 5 genes, including *clfA*, *clfB*, *cna*, *sarA*, and *sigB*, were found in all 65 (100.0%) isolates. The *fnbA*, *fnbB*, *icaA*, *luxS*, *tcaR*, and *ccp* genes were detected in 21 (32.3%), 23 (35.4%), 45 (69.2%), 55 (84.6%), 56

(86.2%), and 62 (95.4%) isolates, respectively (Table 7; Figure 1). The detection frequency of *fnbB* and *icaA* showed a significant difference in isolates from farm A (29.2% and 40.0%, respectively) than those from farm B (6.2% and 29.2%, respectively; $P < 0.05$; Table 7), and the detection frequency of the *luxS* gene displayed a significant difference in isolates between farm A (33.8%) and farm B (50.8%; $P < 0.05$; Table 7).

The microtiter plate assay demonstrated that 64 isolates from the 2 farms could produce biofilm at different intensities (Table 7; Figure 1). Forty isolates (61.5%, 40/65), including 19 from farm A and 21 from farm B, were able to produce weak biofilm; 17 strains

Table 2. Clonal distribution of *Staphylococcus aureus* causing subclinical bovine mastitis in southern Xinjiang, China; CC = clonal complex, ST = sequence type, MLST = multilocus sequence typing

CC (no.)	ST (no.)	MLST allele	<i>spa</i> type	<i>spa</i> repeat succession	Farm
CC81 (12)	ST81 (3)	1-1-1-9-1-1-1	t5100	07-21-16-34-33-13	A
	ST5274 ¹ (1)	1-1-706-9-1-1-1	t5100	07-21-16-34-33-13	A
	ST5290 ¹ (1)	1-1-1-404-1-1-1	t5100	07-21-16-34-33-13	A
	ST5292 ¹ (1)	627-1-1-9-646-1-1	t5100	07-21-16-34-33-13	A
	ST5294 ¹ (1)	1-1-1-9-1-1-703	t5100	07-21-16-34-33-13	A
	ST5301 ¹ (1)	628-782-711-9-1-1-1	t5100	07-21-16-34-33-13	A
	ST5302 ¹ (1)	1-1-712-9-1-1-39	t5100	07-21-16-34-33-13	A
	ST5304 ¹ (1)	1-1-1-405-1-1-1	t5100	07-21-16-34-33-13	A
	ST5305 ¹ (1)	626-1-1-9-1-1-1	t5100	07-21-16-34-33-13	A
	ST5359 ¹ (1)	643-1-1-9-1-1-1	t5100	07-21-16-34-33-13	A
	ST398 (2)	3-35-19-2-20-26-39	t034	08-16-02-25-02-25-34-24-25	A
	ST5291 ¹ (1)	3-35-19-2-20-26-704	t2970	08-16-02-25-34-34-24-25	A
	ST5295 ¹ (1)	633-35-719-2-20-26-39	t034	08-16-02-25-02-25-34-24-25	A
	ST5296 ¹ (1)	3-35-19-2-20-26-705	t2970	08-16-02-25-34-34-24-25	A
	ST5297 ¹ (1)	634-35-19-2-20-26-39	t034	08-16-02-25-02-25-34-24-25	A
CC398 (15)	ST5298 ¹ (1)	159-35-713-2-20-26-39	t034	08-16-02-25-02-25-34-24-25	A
	ST5299 ¹ (1)	630-35-19-2-20-26-704	t034	08-16-02-25-02-25-34-24-25	A
	ST5303 ¹ (1)	635-35-19-2-20-26-39	t034	08-16-02-25-02-25-34-24-25	A
	ST5357 ¹ (1)	644-35-718-2-20-26-39	t034	08-16-02-25-02-25-34-24-25	A
	ST5358 ¹ (1)	3-785-19-2-651-26-39	t034	08-16-02-25-02-25-34-24-25	A
	ST5421 ¹ (1)	3-35-19-2-645-26-39	t034	08-16-02-25-02-25-34-24-25	A
	ST398 (2)	3-35-19-2-20-26-39	t571	08-16-02-25-02-25-34-25	B
	ST5336 ¹ (1)	3-35-723-2-20-26-717	t571	08-16-02-25-02-25-34-25	B
	ST88 (1)	22-1-14-23-12-4-31	t2592	07-12-21-17-13-13-34-34-34-33-13	A
	ST5289 ¹ (1)	22-1-14-400-12-4-700	t2592	07-12-21-17-13-13-34-34-34-33-13	A
	ST5293 ¹ (1)	625-1-14-23-12-587-31	t2592	07-12-21-17-13-13-34-34-34-33-13	A
	ST5300 ¹ (1)	629-1-14-23-12-4-706	t1764	07-12-21-17-13-13-34-34-34-34-33-13	A
	ST5306 ¹ (1)	22-1-709-23-12-4-702	t2592	07-12-21-17-13-13-34-34-34-33-13	A
	ST5361 ¹ (1)	625-1-710-23-12-4-31	t2592	07-12-21-17-13-13-34-34-34-33-13	A
CC5405 (20)	ST5405 ¹ (9)	3-786-1-8-1-1-1	t189	07-23-12-21-17-34	B
	ST5333 ¹ (1)	3-786-1-8-1-1-708	t189	07-23-12-21-17-34	B
	ST5334 ¹ (1)	636-786-322-8-652-1-1	t189	07-23-12-21-17-34	B
	ST5348 ¹ (1)	3-786-720-8-1-1-1	t189	07-23-12-21-17-34	B
	ST5335 ¹ (1)	3-786-1-8-653-1-1	t189	07-23-12-21-17-34	B
	ST5404 ¹ (1)	3-796-727-8-658-1-1	t189	07-23-12-21-17-34	B
	ST5350 ¹ (1)	3-797-1-8-1-1-1	t189	07-23-12-21-17-34	B
	ST5351 ¹ (1)	3-786-728-8-1-1-720	t189	07-23-12-21-17-34	B
	ST5362 ¹ (1)	3-798-729-8-1-595-721	t189	07-23-12-21-17-34	B
	ST5363 ¹ (1)	3-786-1-418-659-1-1	t189	07-23-12-21-17-34	B
	ST5364 ¹ (1)	3-786-638-419-660-1-723	t189	07-23-12-21-17-34	B
	ST5349 ¹ (1)	3-786-1-8-1-1-719	t189	07-23-12-21-17-34	B
CC5406 (12)	ST5406 ¹ (9)	3-16-12-2-13-13-15	t519	04-20-17-25	B
	ST5401 ¹ (1)	3-794-12-2-13-13-716	t519	04-20-17-25	B
	ST5402 ¹ (1)	3-16-12-2-656-13-15	t519	04-20-17-25	B
	ST5403 ¹ (1)	3-16-724-2-13-13-15	t519	04-20-17-25	B

¹New sequence type found in this study.

Table 3. Antibiotic resistance of *Staphylococcus aureus* from subclinical bovine mastitis in dairy farms located in southern Xinjiang, China

Antibiotic class	Antibiotic	Strains, no. (%)
β-Lactams	Penicillin G	38 (58.5)
	Cefoxitin	0 (0)
Glycopeptides	Vancomycin	0 (0)
Aminoglycosides	Gentamicin	8 (12.3)
Macrolides	Erythromycin	29 (44.6)
Tetracyclines	Tetracycline	12 (18.5)
Fluoroquinolones	Ciprofloxacin	3 (4.6)
	Norfloxacin	3 (4.6)
Lincosamides	Clindamycin	26 (40.0)
Folate pathway antagonists	Sulphamethoxazole-trimethoprim	0 (0)
Phenicals	Chloramphenicol	1 (1.5)
Ansamycins	Rifampicin	6 (9.2)
Streptogramins	Quinupudin-dafupudin	4 (6.2)
Oxazolidomones	Linezolid	7 (10.8)
No resistance to antimicrobial agent		21 (32.3)
Resistant to 1 antimicrobial agent		8 (12.3)
Resistant to 2 antimicrobial agents		9 (13.8)
Multi-drug resistant		27 (41.5)

(26.2%, 17/65), including 7 isolates from farm A and 10 from farm B, formed moderate biofilm; and 7 strains (10.8%, 7/65), including 3 from farm A and 4 from farm B, could produce strong biofilm.

DISCUSSION

Contamination of dairy cows and raw milk by *S. aureus*, especially those strains expressing an MDR phenotype and possessing the ability to produce biofilm and toxins, including enterotoxins TSST-1 and PVL, remains an urgent public health issue (Cavicchioli et al., 2015; Wang et al., 2016; Wang et al., 2018). Dairy

products contaminated with *S. aureus* have led to food-borne poisoning outbreaks, demonstrating the public health significance of *S. aureus*. Food-borne infections are frequently reported in China in recent years (Rong et al., 2017; Chen and Xie, 2019; Wu et al., 2019a). Additionally, the economic losses to dairy farms are considerable, because dairy cow mastitis results in decreased milk production, increased health care costs, and higher culling rates (Hennekinne et al., 2012). This study investigated the prevalence, genetic diversity, antimicrobial resistance phenotypes, carriage of staphylococcal virulence genes, and capacity of these isolates to form biofilm. All of these *S. aureus* were isolated from

Table 4. Antimicrobial susceptibility of the study isolates to the 14 antimicrobial agents

Antimicrobial ¹	Resistant, no. of isolates (%)			Intermediate, no. of isolates (%)			Susceptible, no. of isolates (%)		
	Farm A	Farm B	Total	Farm A	Farm B	Total	Farm A	Farm B	Total
FOX	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	30 (46.2)	35 (53.8)	65 (100.0)
C	1 (1.5)	0 (0)	1 (1.5)	3 (4.6)	1 (1.5)	4 (6.2)	26 (40.0)	34 (52.3)	60 (92.3)
TE	11 (16.9)*	1 (1.5)	12 (18.5)	6 (9.2)	5 (7.7)	11 (16.9)	13 (20.0)	29 (44.6)	42 (64.6)
P	21 (32.3)	17 (26.2)	38 (58.5)	0 (0)	0 (0)	0 (0)	9 (13.8)	18 (27.7)	27 (41.5)
LZD	7 (10.8)	0 (0)	7 (10.8)	0 (0)	0 (0)	0 (0)	23 (35.4)	35 (53.8)	58 (89.2)
E	9 (13.8)	20 (30.8)	29 (44.6)	5 (7.7)	0 (0)	5 (7.7)	16 (24.6)	15 (23.1)	31 (47.7)
RD	5 (7.7)	1 (1.5)	6 (9.2)	1 (1.5)	0 (0)	1 (1.5)	24 (36.9)	34 (52.3)	58 (89.2)
CIP	0 (0)	3 (4.6)	3 (4.6)	2 (3.1)	1 (1.5)	3 (4.6)	28 (43.1)	31 (47.7)	59 (90.8)
DA	9 (13.8)	17 (26.2)	26 (40.0)	0 (0)	0 (0)	0 (0)	21 (32.3)	18 (27.7)	39 (60.0)
SXT	0 (0)	0 (0)	0 (0)	1 (1.5)	0 (0)	1 (1.5)	29 (44.6)	35 (53.8)	64 (98.5)
NOR	0 (0)	3 (4.6)	3 (4.6)	0 (0)	0 (0)	0 (0)	30 (46.2)	32 (49.2)	62 (95.4)
CN	6 (9.2)	2 (3.1)	8 (12.3)	0 (0)	0 (0)	0 (0)	24 (36.9)	33 (50.8)	57 (87.7)
QD	4 (6.2)	0 (0)	4 (6.2)	6 (9.2)	6 (9.2)	12 (18.5)	20 (30.8)	29 (44.6)	49 (75.4)
VAN	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	30 (46.2)	35 (53.8)	65 (100.0)

¹FOX = cefoxitin; C = chloramphenicol; TE = tetracycline; P = penicillin G; LZD = linezolid; E = erythromycin; RD = rifampicin; CIP = ciprofloxacin; DA = clindamycin; SXT = sulfamethoxazole-trimethoprim; NOR = norfloxacin; CN = gentamicin; QD = quinupudin-dafupudin; VAN = vancomycin.

* $P < 0.05$.

Table 5. Relationship between molecular typing of *Staphylococcus aureus* and drug resistance¹

Clonal complex (CC; no.)	<i>spa</i> typing (no.)	FOX (%)	C (%)	TE (%)	P (%)	LZD (%)	E (%)	RD (%)	CIP (%)	DA (%)	SXT (%)	NOR (%)	CN (%)	QD (%)	VAN (%)
CC81 (12)	t5100 (12)	0	0	0	58.3	8.3	0	16.7	0	16.7	0	0	0	8.3	0
CC398 (15)	t034 (10)	0	10.0	90.0	100.0	50.0	70.0	30.0	0	50.0	0	0	50.0	20.0	0
	t2970 (2)	0	0	100.0	100.0	0	100.0	0	0	50.0	0	0	50.0	50.0	0
	t571 (3)	0	0	0	100.0	0	100.0	0	33.3	66.7	0	33.3	33.3	0	0
CC88 (6)	t2592 (5)	0	0	0	40.0	20.0	0	0	0	20.0	0	0	0	0	0
	t1764 (1)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC5405 (20)	t189 (20)	0	0	0	15.0	0	25.0	0	0	20.0	0	0	0	0	0
CC5406 (12)	t519 (12)	0	0	8.3	91.7	0	100.0	8.3	16.7	91.7	0	16.7	8.3	0	0
Farm A	30	0	1.5	16.9*	32.3	10.8	13.8	7.7	0	13.8	0	0	9.2	6.2	0
Farm B	35	0	0	1.5	26.2	0	30.8	1.5	4.6	26.2	0	4.6	3.1	0	0
Total	65	0	1.5	18.5	58.5	10.8	44.6	9.2	4.6	40.0	0	4.6	12.3	6.2	0

¹FOX = ceftiofur; C = chloramphenicol; TE = tetracycline; P = penicillin G; LZD = linezolid; E = erythromycin; RD = rifampicin; CIP = ciprofloxacin; DA = clindamycin; SXT = sulfamethoxazole-trimethoprim; NOR = norfloxacin; CN = gentamicin; QD = quinupridin-dafupudin; VAN = vancomycin.

* $P < 0.05$.

raw milk samples taken from 2 dairy farms in southern Xinjiang, China.

In this study, *S. aureus* isolates displayed the highest rate of resistance to penicillin (58.5%). This result is not surprising, because β -lactams are widely prescribed to treat bovine mastitis cases in China, as well as in Turkey (Aslantaş and Demir, 2016). Previous studies performed in China revealed prevalence of penicillin resistance of 94.3% in Ningxia, 80.5% in Shanxi, and 31.3% in Beijing (Li et al., 2015; Wang et al., 2016; Wang et al., 2018). The erythromycin resistance rate (44.6%) found in our study was inconsistent with previous studies conducted by Li et al. (2017) in south China and Wang et al. (2018) in Beijing, who reported resistance rates of 23.6 and 5.2%, respectively. However, Wu et al. (2019b), Wang et al. (2016), and Liu et al. (2017) reported erythromycin resistance rates as 83.3% in 39 Chinese cities, 68.6% in Ningxia, China, and 46.3% in 4 cities of northern China, respectively. The rate of resistance to tetracycline found in our study (18.5%) was higher than those found in Beijing by Wang et al. (2018; 1.0%), in Ningxia by Wang et al. (2016; 5.7%), and in 4 cities of northern China by Liu et al. (2017; 13.0%), but lower than the findings in the Shaanxi Province by Li et al. (2015; 43.9%) and in 39 Chinese cities by Wu et al. (2019b; 65.7%), suggesting that resistance rates for *S. aureus*, as for other bacteria, vary regionally and are influenced by antimicrobial agent usage. Our results in this study support this, as we found a significant difference in tetracycline resistance between isolates from farm A and those from farm B ($P < 0.05$; Tables 4 and 5).

In recent years, the emergence of MDR *S. aureus*, particularly MRSA, has become a growing public health concern (Li et al., 2015). Although no MRSA was detected in our study, the rate of MDR phenotype occurrence (41.5%) was higher than that reported by Li et al. (2017; 23.6%) and Wang et al. (2018; 6.3%) but lower than that reported by Liu et al. (2017; 61.1%). Methicillin-resistant *S. aureus* is considered a major cause of hospital- and community-acquired infections (Gopal and Divya, 2017; Wang et al., 2018). Recently, the isolation of MRSA from raw milk and dairy products has been reported worldwide (Rola et al., 2016; Basanisi et al., 2017; Wu et al. 2019b). The high prevalence of antimicrobial-resistant *S. aureus* from raw milk, the raw material for dairy products, should be considered a potential risk to human health in southern Xinjiang, China.

With respect to the risk of pathogenicity, the presence of virulence genes among all 65 isolates was also evaluated in this study. Staphylococcal HL have been accepted as important virulence factors that contribute to bacterial invasion and escape from the host immune

Figure 1. Antimicrobial susceptibility testing (AST), virulence genes, biofilm-related genes and biofilm production, *mecA* gene, and molecular characterization of 65 *Staphylococcus aureus* isolates cultured from raw milk in southern Xinjiang, China. Sixty-five isolates were grouped into 5 clonal complexes (CC) and 8 *spa* types. The results of AST are shown in different colors according to isolates' diameter of inhibition zone in response to different antimicrobial agents. Blue squares indicate susceptibility, brown squares indicate intermediate response, and black squares indicate resistance. The detections of virulence genes, biofilm-related genes and biofilm production, and *mecA* genes were summarized on a heat map. Green squares denote that the studied genes were detected in those isolates. Red squares denote that those isolates lack the studied genes. ST = sequence type; BFA = biofilm formation ability. Antimicrobial agents used are abbreviated as follows: FOX = ceftiofur; C = chloramphenicol; TE = tetracycline; P = penicillin G; LZD = linezolid; E = erythromycin; RD = rifampicin; CIP = ciprofloxacin; DA = clindamycin; SXT = sulphamethoxazole-trimethoprim; NOR = norfloxacin; CN = gentamicin; QD = quinupridin-dafupridin; VAN = vancomycin.

Table 6. Relationship between molecular typing of *Staphylococcus aureus* and virulence gene detection

Clonal complex (CC; no.)	<i>spa</i> typing (no.)	Virulence gene (%)													
		<i>tsst</i>	<i>pvl</i>	<i>eta</i>	<i>etb</i>	<i>hla</i>	<i>hly</i>	<i>sea</i>	<i>seb</i>	<i>sec</i>	<i>sed</i>	<i>see</i>	<i>seg</i>	<i>seh</i>	<i>sei</i>
CC81 (12)	t5100 (12)	58.3	33.3	0	0	91.7	100.0	8.3	0	16.7	33.3	0	0	91.7	0
CC398 (15)	t034 (10)	0	10.0	0	0	100.0	100.0	10.0	0	0	40.0	0	0	90.0	0
	t2970 (2)	0	0	0	0	100.0	100.0	0	0	0	50.0	0	0	100.0	0
	t571 (3)	0	33.3	0	0	100.0	100.0	0	0	66.7	33.3	0	0	0	66.7
CC88 (6)	t2592 (5)	0	0	0	0	100.0	100.0	20.0	0	0	40.0	0	0	80.0	0
	t1764 (1)	0	0	0	0	100.0	100.0	0	0	0	0	0	0	100.0	0
	t189 (20)	0	50.0	0	0	100.0	100.0	0	0	65.0	25.0	0	10.0	0	65.0
CC5405 (20)	t519 (12)	83.3	83.3	0	0	91.7	100.0	0	0	41.7	8.3	0	0	0	100.0
Farm A	30	10.8	7.7	0	0	44.6	46.2	4.6	0	3.1	16.9	0	0	41.5	0
Farm B	35	15.4	32.3*	0	0	52.3	53.8	0	0	30.8*	10.8	0	3.1	0	41.5
Total	65	26.2	40.0	0	0	96.9	100.0	4.6	0	33.8	27.7	0	3.1	41.5	7.7

* $P < 0.05$.**Table 7.** Relationship between molecular typing of *Staphylococcus aureus* and biofilm formation genes and biofilm formation ability (BFA)

Clonal complex (CC; no.)	<i>spa</i> typing (no.)	Biofilm-related genes (%)														BFA ¹ (%)			
		<i>fmbA</i>	<i>fmbB</i>	<i>clfA</i>	<i>clfB</i>	<i>cna</i>	<i>icaA</i>	<i>sarA</i>	<i>bap</i>	<i>tcaR</i>	<i>ccp</i>	<i>luxS</i>	<i>agrC</i>	<i>sigB</i>		–	+	++	+++
CC81 (12)	t5100 (12)	16.7	16.7	100.0	100.0	100.0	75.0	100.0	0	91.7	100.0	75.0	0	100.0	0	8.3	58.3	33.3	0
CC398 (15)	t034 (10)	40.0	90.0	100.0	100.0	100.0	100.0	100.0	0	90.0	90.0	80.0	0	100.0	0	0	80.0	20.0	0
	t2970 (2)	50.0	100.0	100.0	100.0	100.0	100.0	100.0	0	100.0	100.0	100.0	0	100.0	0	0	50.0	50.0	0
	t571 (3)	33.3	100.0	100.0	100.0	100.0	66.7	100.0	0	100.0	100.0	100.0	0	100.0	0	0	100.0	0	0
CC88 (6)	t2592 (5)	0	20.0	100.0	100.0	100.0	20.0	100.0	0	60.0	80.0	40.0	0	100.0	0	0	20.0	20.0	60.0
	t1764 (1)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	0	100.0	100.0	100.0	0	100.0	0	0	100.0	0	0
	t189 (20)	45.0	5.0	100.0	100.0	100.0	65.0	100.0	0	80.0	100.0	95.0	0	100.0	0	0	60.0	25.0	15.0
CC5406 (12)	t519 (12)	16.7	0	100.0	100.0	100.0	33.3	100.0	0	91.7	91.7	91.7	0	100.0	0	0	58.3	33.3	8.3
Farm A	30	13.8	29.2*	46.2	46.2	46.2	40.0*	46.2	0	40.0	43.1	33.8	0	46.2	0	1.5	27.7	12.3	4.6
Farm B	35	18.5	6.2	53.8	53.8	53.8	29.2	53.8	0	46.2	52.3	50.8*	0	53.8	0	0	33.8	13.8	6.2
Total	65	32.3	35.4	100.0	100.0	100.0	69.2	100.0	0	86.2	95.4	84.6	0	100.0	0	1.5	61.5	26.2	10.8

¹BFA: – = negative; + = weak; ++ = moderate; +++ = strong.* $P < 0.05$.

response (Pereyra et al., 2016; Wang et al., 2016). Our study showed that 96.9% of the total isolates possessed the *hla* gene, and 100.0% of strains had the *hly* gene, in agreement with previous reports (Wang et al., 2016; Li et al., 2017). Toxic shock syndrome toxin-1 (*tsst-1*), a superantigen secreted by *S. aureus* in infected hosts, is responsible for toxic shock syndrome in humans (Udo et al., 2016; Wang et al., 2016). Our findings showed that 26.2% of *S. aureus* isolates harbored the *tsst-1* gene. The prevalence of the *tsst-1* gene in our study was higher than that reported by Wang et al. (2018; 2.1%) and Liu et al. (2017; 14.8%) but lower than that reported by Wang et al. (2016; 40.0%). Panton-Valentine leukocidin plays an important role in destroying host leukocytes and results in tissue necrosis (Wang et al., 2018). In the present study, 40.0% of *S. aureus* isolates had the *pvl* gene, which was lower than that (93.8%) reported in a previous study (Wang et al., 2018) and higher than that reported by Liu et al. (2017; 14.8%). Interestingly, the prevalence rate of the *pvl* gene in samples from farm B (32.3%) was higher than in those from farm A (7.7%; $P < 0.05$). The classic enterotoxin genes of *S. aureus* (SE) are known to be responsible for food poisoning incidents. It has been reported that more than 80.0% of isolates from cow milk related to mastitis carried 1 or more SE genes (Song et al., 2015; Wang et al., 2018). In this study, 95.4% of isolates harbored SE-encoding genes, and *seh* and *sei* (each 41.5%) were the most frequently detected enterotoxin genes. This finding is different from those in previous reports from China and Australia (McMillan et al., 2016; Liu et al., 2017; Wang et al., 2018). Additionally, the prevalence rate of the *sec* gene from farm B (30.8%) was higher than that from farm A (3.1%; $P < 0.05$). Therefore, the different prevalence rates of SE genes in *S. aureus* may be attributable to the fact that these isolates originated in geographically diverse locations. According to previous reports, the *see* gene was rarely present in raw milk or even retail food in China (Liu et al., 2017; Wang et al., 2018), and similarly, this marker was absent in this study.

The biofilm-producing ability of staphylococci has increasingly been considered an important virulence trait. Also, staphylococcal adhesins have been shown to be essential to bind host cells. Therefore, this study also investigated the distribution of biofilm-related and adhesin genes among all isolates. The *fmbA* gene was detected in 32.3% of *S. aureus* isolates, which is distinct from the findings of Aslantaş and Demir (2016; 77.7%), Wang et al. (2016; 94.3%), and Li et al. (2017; 94.3%). The prevalence rate of the *fmbB* gene in our study was 35.4%, which is lower than that reported by Wang et al. (2016; 80.0%). Additionally, the prevalence rate of the *fmbB* gene from farm A (29.2%) was higher than

that from farm B (6.2%; $P < 0.05$). The *clfA* gene was detected in all 65 isolates, which is consistent with a previous study conducted by Li et al. (2017) in southern China. In contrast, the *bap* and *argC* genes were absent in all 65 isolates. The prevalence rate of the *icaA* gene from farm A (40.0%) was higher than that from farm B (29.2%; $P < 0.05$), whereas the prevalence rate of the *luxS* gene from farm B (50.8%) was higher than that from farm A (33.8%; $P < 0.05$). The different prevalence rates of these genes in *S. aureus* could be attributable to the fact that these tested isolates originated in geographically diverse locations. In the present study, 64 isolates could form biofilms, as measured by the microtiter plate assay described above, and these findings agree with a previous study conducted in Beijing (Wang et al., 2018). The high incidence of biofilm-forming *S. aureus* isolates in these studies suggests the necessity for dairy farms to improve quality-assurance systems, to decrease and eliminate these isolates (Wang et al., 2018).

Our data also highlighted the genetic diversity of the *S. aureus* isolates from subclinical mastitis, through MLST and *spa* typing. In the present study, the 65 *S. aureus* isolates from 2 geographic regions in southern Xinjiang, China, were grouped into 44 ST and 8 *spa* types. Importantly, analysis of the isolates in this study identified 41 novel ST not previously reported (Table 2; Figure 2). The goeBURST algorithm was used to examine the population structure of isolates in our study relative to global bovine isolates. The global population structure of bovine milk-associated *S. aureus* ST formed 14 primary groups (McMillan et al., 2016). With the exception of ST5405 and ST5406, bovine isolates from this study were representative of the predicted founding ST of their associated group or CC. Twelve of the bovine isolates from this study fell into CC81, 15 into CC398, 6 into CC88, 20 into CC5405, and 12 into CC5406. The isolates ST5405 and ST5406 have emerged as dominant lineages derived from bovine milk in this study, which is distinct from findings of Hata et al. (2010) and McMillan et al. (2016).

CONCLUSIONS

Our study demonstrated that *S. aureus* isolates of subclinical bovine mastitis in southern Xinjiang, China, widely harbored both biofilm-related and enterotoxin genes involved in the pathogenesis of *S. aureus* infections, which indicate potential virulence of the isolates. Additionally, high levels of resistance against β -lactams and erythromycin, which are widely used in veterinary practice, were observed in this study. More importantly, a high percentage of MDR was present in this study, so the pathogenicity and transmission

Figure 2. goeBURST diagram for the multilocus sequence typing (MLST) data of 65 *Staphylococcus aureus* isolates. The goeBURST diagram was calculated using PHYLOViZ with the goeBURST algorithm (<http://goeBURST.phyloviz.net>). Sequence types (ST) were grouped according to their allelic profiles. All 44 ST are represented by filled circles. Each filled circle represents a unique ST. Central circles represent ST that are group founders of MLST clonal complexes. All other ST are single-locus variants or multi-loci variants. Light blue circles represent new ST identified in this study.

risk of *S. aureus* cannot be ignored. Notably, it is essential to control and eliminate the presence of MDR, enterotoxigenic, and biofilm-forming *S. aureus* in raw milk. Also, our study showed the genetic diversity of these isolates. Forty-one new ST were detected for the first time. Significant differences in genetic diversity, along with antibiotic resistance and biofilm formation, were observed for *S. aureus* isolates from subclinical mastitis. Our study showed that *S. aureus* strains with similar genetic characteristics exhibited specific antibiotic resistance patterns, virulence gene profiles, and biofilm formation abilities. Therefore, monitoring the genotypes of *S. aureus* in dairy herds would help distinguish prevalent clones, to develop control strategies for mastitis caused by *S. aureus*.

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ORCIDS

- Qiang Ren <https://orcid.org/0000-0001-6323-6465>
 Guanhua Liao <https://orcid.org/0000-0002-3895-0621>
 Zihao Wu <https://orcid.org/0000-0002-0241-1220>
 Junfan Lv <https://orcid.org/0000-0002-5937-9751>
 Wei Chen <https://orcid.org/0000-0002-3761-1572>