Antimicrobial Resistance and Molecular Typing of *Staphylococcus aureus* Isolates from Raw Milk in Hunan Province

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Background. *Staphylococcus aureus* is one of the most important foodborne pathogens in the world and the main cause of dairy cow mastitis. Few studies have investigated the epidemic pedigree of *S. aureus* of bovine origin in Hunan, China. Therefore, we aimed to analyze the capsular polysaccharides (CP), molecular typing, and antibiotic resistance characteristics of *S. aureus* isolated from raw milk of dairy farms in Hunan Province. **Methods.** Between 2018 and 2022, 681 raw milk samples were collected from dairy cows from farms in Changsha, Changde, Shaoyang, Yongzhou, and Chenzhou in Hunan Province. *S. aureus* was isolated from these samples, and the isolates were subjected to molecular typing, CP typing, and determination of antibiotic resistance through broth dilution and polymerase chain reaction (PCR). **Results.** From 681 raw milk samples, 76 strains of *S. aureus* were isolated. The pathogenicity of 76 isolates was determined preliminarily by detecting *cp5* and *cp8* CP genes. Eighteen types of antimicrobial resistance phenotypes of 76 *S. aureus* strains were detected by the broth dilution method, and 11 kinds of related resistance genes were amplified by PCR. The *S. aureus* isolates had CP5 (42.10%) and CP8 (57.89%). *S. aureus* had a multiple antimicrobial resistance rate of 26.75%. The isolated strains had the highest resistance rate to penicillin (82.89%) and showed varying degrees of resistance to other drugs, but no isolate showed resistance to doxycycline. The 76 isolates all carried two or more antibiotic resistance genes, with a maximum of eight antibiotics resistance genes. *FemB* was detected in all isolates, but none of isolates carried *vanA*, *ermA*, or *glrA.* The 76 isolates were divided into 22 sequence types (ST) and 20 *spa* types by MLST and *spa* typing, and the number of t796-ST7 (n=15) isolates was the highest, which may be the major epidemic strain of multidrug-resistant *S. aureus*. **Conclusion.** The present findings indicate the need to increase production of the CP8 *S. aureus* vaccine in Hunan Province and strengthen resistance monitoring of t796-ST7

isolates with the prevalent molecular type of multi - drug resistant strains. The use of β-lactam, macrolides, and lincosamides should be reduced; doxycycline, sulfonamides, and glycopeptides could be appropriately added to veterinary antibiotics to treat infectious diseases in dairy cows.

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Abstract

Background. *Staphylococcus aureus* is one of the most important foodborne pathogens in the world and the main cause of dairy cow mastitis. Few studies have investigated the epidemic pedigree of *S. aureus* of bovine origin in Hunan, China. Therefore, we aimed to analyze the capsular polysaccharides (CP), molecular typing, and antibiotic resistance characteristics of *S. aureus* isolated from raw milk of dairy farms in Hunan Province.

Methods. Between 2018 and 2022, 681 raw milk samples were collected from dairy cows from farms in Changsha, Changde, Shaoyang, Yongzhou, and Chenzhou in Hunan Province. *S. aureus* was isolated from these samples, and the isolates were subjected to molecular typing, CP typing, and determination of antibiotic resistance through broth dilution and polymerase chain reaction (PCR).

Results. From 681 raw milk samples, 76 strains of *S. aureus* were isolated. The pathogenicity of 76 isolates was determined preliminarily by detecting *cp5* and *cp8* CP genes. Eighteen types of antimicrobial resistance phenotypes of 76 *S. aureus* strains were detected by the broth dilution method, and 11 kinds of related resistance genes were amplified by PCR. The *S. aureus* isolates had CP5 (42.10%) and CP8 (57.89%). *S. aureus* had a multiple antimicrobial resistance rate of 26.75%. The isolated strains had the highest resistance rate to penicillin (82.89%) and showed varying degrees of resistance to other drugs, but no isolate showed resistance to doxycycline. The 76 isolates all carried two or more antibiotic resistance genes, with a maximum of eight antibiotics resistance genes. *FemB* was detected in all isolates, but none of isolates carried *vanA*, *ermA*, or *glrA*. The 76 isolates were divided into 22 sequence types (ST) and 20 *spa* types by MLST and *spa* typing, and the number of t796-ST7 (n=15) isolates was the highest, which may be the major epidemic strain of multidrug-resistant *S. aureus*.

39 **Conclusion.** The present findings indicate the need to increase production of the CP8 *S. aureus*
40 vaccine in Hunan Province and strengthen resistance monitoring of t796-ST7 isolates with the
41 prevalent molecular type of multidrug resistant strains. The use of β-lactam, macrolides, and
42 lincosamides should be reduced; doxycycline, sulfonamides, and glycopeptides could be
43 appropriately added to veterinary antibiotics to treat infectious diseases in dairy cows.

Introduction

45 *Staphylococcus aureus* has long been a threat to human health and the development of animal
46 husbandry. In many countries, 30% to 40% cases of bovine mastitis are caused by *S. aureus*
47 (Basanisi et al., 2017), mainly through adhesion, which leads to colonization in the mammary
48 epithelial cells of livestock and results in udder abscesses, hardening, fistulae, necrosis, and
49 exfoliation. Diseased bovines present with obvious udder fever, udder hardness and pain, and
50 reddish to black milk with foul-smelling flocs, which not only reduces milk production and
51 quality, but also endangers the animal's life (Hiramatsu et al., 2002; Sinha & Fraunholz, 2010).
52 Non-standard processing and disinfection procedures can easily result in the production of dairy
53 products contaminated with *S. aureus* (Schmidt et al., 2017; Johler et al., 2018), and many types
54 of *S. aureus* can be detected in raw dairy products worldwide (Alghizzi & Shami, 2021). *S.*
55 *aureus* causes infection in the host body by releasing a variety of toxins, the most important
56 being Panton–Valentine leukocidin (PVL). Encoded by *lukF PV* and *lukS PV*, PVL is a poor-
57 forming toxin that causes host immune suppression and infection by attacking leukocytes, such
58 as neutrophils and monocytes (Spaan et al., 2017); *S. aureus* exfoliative toxin (Ets) causes severe
59 inflammation of the gastrointestinal tract by stimulating intestinal epithelial cells (Banwell et al.,
60 1973). Toxic shock syndrome toxin can promote activation of T lymphocytes and enhance the
61 secretion of inflammatory factors, resulting in a severe immune response of the animal body with
62 subsequent multiple organ failure (Hoppe et al., 2018). Ets reduces the adhesion of mammalian
63 stratum corneum cells by acting on desmosomal core glycoproteins between surface granular
64 cells, resulting in the loosening of keratinocytes, which leads to the staphylococcal scalded-skin
65 syndrome. Bacteria enter the bloodstream, leading to septicemia and endocarditis as the
66 immunity of the infected host decreases (Rodríguez-Lázaro et al., 2015). Recently, foodborne *S.*
67 *aureus* infections have been frequently reported in China (Wu et al., 2019). In 2015, microbial
68 food poisoning accounted for 53.7% of all food poisoning emergencies in China, and *S. aureus*
69 was the main pathogen (Wu et al., 2018). *S. aureus* not only has a great impact on the dairy
70 industry but also poses a threat to public health and food safety.
71 The invasiveness of *S. aureus* depends on its resistance to host phagocytes and its cell adhesion.
72 The capsular polysaccharide (CP) of *S. aureus* is an important factor related to its anti-
73 phagocytosis and cell adhesion properties, and it can be classified into 11 serotypes based on its
74 antigenic structure (Sompolinsky et al., 1985). CP1 and 2 are rarely encountered in clinical
75 practice, and approximately 70% to 80% of *S. aureus* can produce CPs of serotypes 5 and 8,
76 referred to as CP5 and CP8, respectively (Goerke et al., 2005). CP5 and CP8 are expressed by
77 the *cp5* and *cp8* genes, respectively. *Cp5* and *cp8* are nucleic acid sequences of approximately
78 17.5 kb present on bacterial chromosomes. The structures of CP5 and CP8 are 14-3-O-Ac-β-D-

79 Man-NAcA-(1-4)-lFucNAc-(1-3)-β-D-FucNAc-(1-)n and (3)-4-O-Ac-β-D-ManNAcA-(1-3)-L-
80 FucNAc-(1-3)-β-D-FucNAc-(1-), respectively. The structures of CP5 and CP8 mainly differ in
81 terms of the position of sugar linkage and acetylation of amino mannituronic acid residues. It has
82 been shown that CP5 and CP8 are the main factors associated with *S. aureus*-induced mastitis
83 (Poutrel et al., 1988), and serotyping of *S. aureus* CPs is of significance to the local dairy
84 industry to better understand the epidemiology of *S. aureus*.
85 At present, the main method for preventing and treating dairy cow mastitis caused by *S. aureus* is
86 udder infusion with antibiotics. However, with the extensive use of antibiotics, *S. aureus* has
87 acquired resistance to a variety of antibiotics by changing its phenotype, regulating metabolic
88 pathways, and horizontally transferring resistant plasmids. Methicillin-resistant *S. aureus*
89 (MRSA) has spread across the globe since its discovery. Despite its susceptibility to new
90 semisynthetic β-lactams, MRSA is resistant to a variety of antibiotics, such as macrolides,
91 aminoglycosides, and tetracyclines (Hassoun et al., 2017). In the 1990s, MRSA infection,
92 acquired immune deficiency syndrome, and hepatitis B were considered the three most difficult
93 diseases to treat worldwide (Nada et al., 1996). In the animal husbandry industry, MRSA was
94 first isolated from dairy cows with mastitis in 1972 (Devriese et al., 1972), and cows have
95 become its natural hosts (Spohr et al., 2011). The penicillin-binding protein (PBP) 2a encoded by
96 *mecA* is the main cause for MRSA, and related studies have indicated that the detection of *mecA*
97 is the main criterion for identifying MRSA (Kobayashi et al., 1994). Oxacillin-sensitive *mecA*-
98 positive *S. aureus* (OS-MRSA) has been detected in human clinical and animal products
99 (Conceição et al., 2015). Although OS-MRSA carries *mecA*, it is often misidentified as
100 methicillin-sensitive *S. aureus* (MSSA) (Quijada et al., 2019) and is likely to become a subgroup
101 with high resistance under the pressure of β-lactam selection (Martineau et al., 2000).
102 Vancomycin is considered the last line of defense against Gram-positive bacteria and is used to
103 treat infections that do not respond to other antibiotics. However, vancomycin-resistant *S. aureus*
104 (VRSA) has become increasingly common in China and abroad (Haseeb et al., 2019). The
105 resistance of *S. aureus* to vancomycin is usually mediated by the *vanA* operon on transposable
106 Tn1546, which results from the transfer of resistant plasmids from vancomycin-resistant
107 enterococci (Arthur et al., 1993; Lazaris et al., 2017). Under the combined action of *vanA* and
108 *vanH* expression products, d-Lac replaces d-Ala in the cell wall peptidoglycan precursor peptide
109 to form d-Ala-d-Lac, which alters the vancomycin binding site and leads to drug failure (Bugg et
110 al., 1991). As the incidence of multidrug resistance has increased all over the world and is
111 considered a significant public health threat, the proper use of antibiotics has become even more
112 necessary. Moreover, the emergence of multidrug-resistant (MDR) and extensively drug-resistan
113 t (XDR) strains greatly increases the risk of the resistance spreading to humans and having an im
114 pact on the effectiveness of current antibiotic treatments; in fact, this is often a major factor in th
115 e failure of treatment and infection control (Algammal et al., 2020a; Helmy et al., 2018). Additio
116 nally, studies have shown that MDR MRSA isolated from milk carries a variety of major virulen
117 ce factors, such as *sea* and *coa*, that can lead to food poisoning in consumers who ingest the cont
118 aminated milk (Algammal et al., 2020b). The misuse of antibiotics is an important factor leading

119 to the emergence of MDR and XDR pathogens that threaten human public health as well as livest
120 ock-derived and aquatic products (Algammal et al., 2022). Therefore, effective and rigorous mon
121 itoring methods need to be established to control their growth in humans and animals (Algammal
122 et al., 2023) and for routine application of antimicrobial susceptibility testing to detect the antibio
123 tic of choice and screen emerging resistant strains. In this context, monitoring the antimicrobial
124 resistance of *S. aureus* and prevalence of MRSA and VRSA in raw milk is of great significance
125 for the research and development of antibiotics and for identifying the most appropriate drug
126 treatment for animal diseases from the perspective of food safety.
127 Multilocus sequence typing (MLST) is based on the technology of multilocus enzyme
128 electrophoresis. It involves seven pairs of relatively conservative housekeeping genes in the
129 bacterial genome that are sequenced directly and uploaded to an international public database for
130 analysis, which greatly improves the accuracy of multilocus enzyme electrophoresis (Maiden et
131 al., 1998). In addition to the advantages of general genotyping methods, MLST has high
132 resolution and repeatability, making it more convenient and accurate than general genotyping
133 methods (Tivendale et al., 2009). More importantly, MLST overcomes the space restrictions of
134 laboratories, regions, and countries, helps share typing data through large international databases,
135 and enables analysis of the phylogeny, population structure, biological evolution, epidemiology,
136 and molecular genetic evolution of pathogenic microorganisms (van Wamel et al., 2010). *spa*
137 typing is used to sequence the X variable region gene of *S. aureus* protein A; it also reveals
138 changes in base pair numbers, sequence of X region repeat succession, and actions, such as base
139 insertion and deletion. The X region is polymorphic; therefore, the isolates are typed according
140 to the polymorphism of this region (Moosavian et al., 2020). This typing method based on
141 single-base sequence analysis has a standardized international nomenclature and can be
142 integrated into the international *spa* database, which is convenient for exploring molecular
143 correlations between *S. aureus* from different regions, environments, and sources (Rodriguez et
144 al., 2015). *spa* typing can usually be mapped to MLST in the form of *spa*-MLST-mapping
145 (Strommenger et al., 2008), which has become the main method for typing *S. aureus*. It makes
146 the research results highly reproducible and the results of regional and international *S. aureus*
147 pedigree monitoring and source tracing interchangeable (Gurjar et al., 2012).
148 Consumption of contaminated milk is an important way by which *S. aureus* can infect humans,
149 and several cases of bacterial food poisoning related to *S. aureus* have been reported (Fetsch et
150 al., 2014). The fact that raw milk is particularly vulnerable to *S. aureus* contamination, coupled
151 with the increasingly serious antimicrobial resistance of *S. aureus,* makes it a potential threat to
152 the development of the dairy industry and public health safety. Milk forms a significant
153 component of the Chinese balanced diet standards, and the consumption of milk is increasing
154 annually (Qian et al., 2011). As the dairy industry in Hunan Province has advanced, large dairy
155 farms have been built in many cities and counties. However, few studies have examined *S.*
156 *aureus* isolates from raw milk in Hunan. The purpose of this study was to determine the
157 antibiotic resistance, serotype distribution, and molecular epidemic characteristics of *S. aureus*
158 isolated from raw milk of dairy cows in Hunan Province and provide a theoretical basis for

159   rational use of antibiotics in dairy cows in Hunan. These findings could help promote animal
160   welfare and food safety management through rational drug use in the dairy industry.

161   ## Materials & Methods

162   ### Sample collection
163   Between 2018 and 2022, 681 raw milk samples were collected from dairy cows from farms in
164   Changsha, Changde, Shaoyang, Yongzhou, and Chenzhou in Hunan Province. The sample
165   collection was approved by the Ethics Committee of Hunan Agricultural University (no.
166   2022092389). Dairy cow teats were disinfected with cotton balls containing 75% alcohol before
167   milk collection, and the first three handfuls of milk were discarded. All samples were transferred
168   to sterile tubes and transported to the laboratory for bacterial analysis at 4°C.

169   ### Isolation and identification of *S. aureus*
170   One milliliter of the collected raw milk sample was added to 9 mL of 10% sodium chloride
171   trypticase soy broth (Luqiao Bio-Technology Co., Ltd., Beijing, China) and incubated at 36°C
172   for 24 h. An aseptic inoculation ring was dipped in the culture, and chromogenic *S. aureus* agar
173   plates (Hope Bio-Technology Co., Ltd, Qingdao, China) were inoculated and incubated at 36°C
174   for 18 h. Colonies suspected to be of *S. aureus* based on their morphology were selected and
175   subcultured in nutrient agar medium (Huankai Bio-Technology Co., Ltd, Guangdong, China) at
176   36°C for 24 h and subsequently incubated at 36°C for 18 h. Individual colonies on nutrient agar
177   were selected, and catalase tests, Gram staining, matrix-assisted laser desorption ionization-time
178   of flight mass spectrometry (Bruker Daltonics, Karlsruhe, Germany), and VITEK-2 Compact
179   testing (bioMérieux, Marcy-l'Étoile, Lyon, France) were performed. All strains were stored in
180   20% glycerin broth at -80°C. The antibiotic-sensitive quality control strain *S. aureus* ATCC
181   29213 was kindly provided by the Hunan Provincial Institute of Veterinary Drugs and Feed
182   Control.

183   ### Molecular identification of the isolates
184   Genomic deoxyribonucleic acid (DNA) was extracted using a bacterial DNA extraction kit
185   (Bioer Bio-Technology Co. Ltd., Hangzhou, China) according to the manufacturer's instructions.
186   The isolates were confirmed by polymerase chain reaction (PCR) using a primer pair of the
187   thermonuclease gene (*nuc*) (Table 1) and 2 × Phanta Max Master Mix (Dye Plus)(Vazyme
188   Biotech Co., Ltd, Nanjing, China). The amplified PCR products were separated by 1.0% agarose
189   gel electrophoresis. Then, the PCR products of five strains were randomly selected and
190   sequenced, and the sequencing results were compared with those on the National Center for
191   Biotechnology Information database (http://blast.ncbi.nlm.nih.gov).

192   ### Typing of *S. aureus* CPs
193   CP5 (*cp5*) and CP8 (*cp8*) genes were amplified using PCR. The primer sequences, amplicon
194   sizes, and annealing temperatures of *cp5* and *cp8* are shown in Table 1.

195   ### Determination of phenotypic antimicrobial resistance
196   The antimicrobial resistance phenotypes (n=18) of the *S. aureus* isolates were detected using the
197   broth dilution method. Resistance to the following antimicrobial agents was evaluated: β -lactam

198   antibiotics (penicillin, benzoxicillin, cefoxitin, ceftiofurme, and ogmentine); macrolides
199   (erythromycin and timicacin); lincoamines (clindamycin); quinolones (enrofloxacin and
200   ofloxacin); tetracycline (doxycycline); aminoglycosides (gentamicin); sulfonamides
201   (sulfaisoxazole and compound xazole); glycopeptides (vancomycin); and those of other classes
202   (taymycin and linezolid). Circular or diffuse deposits were observed in the wells with bacterial
203   growth, while no turbidity was observed in the liquid in the negative control wells. Antimicrobial
204   resistance was evaluated based on the minimum inhibitory concentration of an antibacterial drug
205   according to the standard values published by the Clinical and Laboratory Standards Institute in
206   2022 (https://clsi.org/). *S. aureus* ATCC 29213 was used for quality control in the drug
207   sensitivity test (Jinzhang Technology Development Co., Ltd., Tianjin, China). As described by
208   Magiorakos et al. (2012), the isolates were classified as MDR (resistant to three of the
209   aforementioned categories of drugs), XDR (only sensitive to class 1 and 2 antibiotics) and
210   pandrug-resistant (PDR, resistant to all types of antibiotics).

211   **PCR amplification of antimicrobial resistance genes**
212   Antimicrobial resistance genes were detected using purified *S. aureus* DNA. The genes for β-
213   lactam (*mecA*, *femA*, *femB*), macrolide (*ermA*, *ermB*, *ermC*), tetracycline (*tetM*), aminoglycoside
214   (*aacA-aphD*), quinolone (*glrA*), phenicol (*flor*), and vancomycin (*vanA*) resistance were
215   amplified using PCR. The resistance gene primers and annealing temperatures are listed in Table
216   1.

217   **MLST and cluster analysis**
218   According to the distribution of alleles and sequence types (STs) in the MLST database
219   (https://pubmlst.org/organism s/staphylococcus-aureus/primers), seven housekeeping genes of *S.*
220   *aureus* were selected for PCR amplification: carbamate kinase (*arcC*), shikimate dehydrogenase
221   (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*),
222   triosephosphate isomerase (*tpi*), and acetyl coenzyme A acetyltransferase (*yqiL*). The sizes of
223   each gene fragment varied from 400 to 520 bp. The gene primers, amplicon sizes, and annealing
224   temperatures are shown in Table 1. All PCR products were sequenced, and the sequencing
225   results were submitted to the MLST database (https://pubMLST.org/) to obtain the
226   corresponding allelic profiles and STs. Cluster analysis was conducted using the PHYLOViZ
227   online tool (https://online.phyloviz.net/index) and BioNumerics software (version 7.6; Applied-
228   Maths, Sint-Martens-Latem, Belgium).

229   ***spa* typing**
230   *spa* types of all isolates were assayed using PCR according to the *spa* primers provided in the
231   Ridom *Spa*Server database (https://*spa*.ridom.de/) (Table 1). All PCR products were sequenced,
232   the sequence number of the repeat succession was analyzed using Ridom *Spa*Server, and the *spa*
233   types of the isolates were obtained.

234   **Simpson's index of diversity calculation**
235   The MLST and *spa* types were inputted into the Comparing Partitions online tool
236   (http://www.comparingpartitions.info/index.php?link=Tool) to calculate the Simpson's index of
237   diversity (Sun et al., 2019).

238 **Statistical analyses**
239 The K independent samples method and a non-parametric test were used to identify the
240 correlation between MLST and *spa* typing, antibiotic resistance genes and phenotypes, and to
241 differentiate between the MRSA and molecular types. Statistical analyses were performed using
242 IBM SPSS Statistics for Windows, version 23.0 (IBM Corp., Armonk, NY, USA).($p<0.05$ was
243 considered to indicate statistical significance; $p>0.1$ was not statistically significant)

244 # Results
245 Determining the main prevalent molecular types of *S. aureus* and their antibiotic resistance is
246 necessary to formulate effective antibiotic treatment strategies and reduce the impact of *S. aureus*
247 infections on the dairy industry and public health. We therefore examined the CPs, molecular
248 types, and antibiotic resistance characteristics of *S. aureus* isolated from raw milk of dairy farms
249 in Hunan Province.
250 **Isolation and identification of *S. aureus* from raw milk**
251 Seventy-six strains (11.16%) of suspected *S. aureus* were isolated from 681 raw milk samples
252 collected from dairy herds in five regions of Hunan Province from 2018 to 2022. The isolation
253 rates of *S. aureus* for samples from Changsha, Changde, Shaoyang, Chenzhou, and Yongzhou
254 were 15.78% (12/76), 44.73% (34/76), 32.89% (25/76), 3.94% (3/76), and 2.63% (2/76),
255 respectively. All isolates appeared as smooth, raised, purplish-red, round colonies on
256 chromogenic medium and smooth, golden-yellow, round colonies on nutrient agar, ranging in
257 size from a pinhead to grain of rice, with positive catalase test results. Gram-positive strains
258 arranged in the shape of grapes were observed on microscopic examination. *Nuc* could be
259 detected in all isolates, and the sequence homology between the PCR products and published
260 strains was 99.21% to 99.23%. Matrix-assisted laser desorption ionization-time of flight mass
261 spectrometry and VITEK-2 Compact testing revealed that the isolates were of *S. aureus*. The
262 biochemical reactions and their results are shown in Table 2 . Based on the above results, the 76
263 isolates were consistent with the biochemical characteristics of *S. aureus* and were identified as
264 *S. aureus*.
265 **CP types of *S. aureus***
266 All isolates were classified as having CP5 or CP8. The detection rates of CP5 for samples from
267 Changsha, Changde, Shaoyang, Yongzhou, and Chenzhou were 75% (9/12), 35.29% (12/34),
268 40% (10/25), 0.00% (0/2), and 33.33% (1/3), respectively, while those of CP8 were 25% (3/12),
269 64.70% (22/34), 60% (15/25), 100% (2/2), and 66.66% (2/3), respectively (Fig. 1).
270 **Phenotypic characteristics of the isolates**
271 The resistance of the 76 *S. aureus* strains to 18 antibiotics was tested using the broth dilution
272 method. The resistance breakpoints of the 18 antibiotics and positive antibiotic resistance results
273 are shown in Table 3. We found that 88.15% (67/76) of the isolates were resistant to at least one
274 antibiotic, 32.89% (25/76) were resistant to three or more antibiotics (MDR strains), and the
275 highest resistance was found among isolates that were resistant to six antibiotic classes. Nine
276 isolates were not resistant to any antibiotic. All isolates were the most resistant to penicillin, with
277 82.89% showing penicillin resistance, followed by oxacillin (25.00%), erythromycin (22.37%),

278   clindamycin (22.37%), tilmicosin (21.05%), gentamicin (19.73%),

279   trimethoprim/sulfamethoxazole (10.53%), florfenicol (7.89%), ofloxacin (6.58%),

280   amoxicillin/clavulanate (3.95%), enrofloxacin (3.95%), sulfisoxazole (3.95%), ceftiofur (2.63%),

281   tiamulin (2.63%), and linezolid (2.63%). One isolate (1.32%) was resistant to vancomycin and

282   was identified as VRSA. All isolates were sensitive to doxycycline. In this study, a total of 21

283   isolates were resistant to three or more classes of antibiotics, and the multiple resistance rate was

284   27.63%. The isolates were resistant to six kinds of antibiotics at most. No isolates sensitive to

285   class 1 or 2 drugs or resistant to all antibiotics were detected. Therefore, the 21 multi-resistant

286   strains could only be classified as MDR, and no isolates could be classified as XDR or PDR. The

287   drug resistance profiles of all MDR isolates are shown in Table 4.

288   **Antibiotic resistance genes of the isolates**

289   All 76 strains carried two or more antibiotic resistance genes, with a maximum of eight genes.

290   All isolates carried *femB* with a detection rate of 100%, followed by *ermB* (80.26%), *aacA-aphD*

291   (76.32%), *femA* (75.00%), *ermC* (68.42%), *mecA* (47.37%), *flor* (42.11%), and *tetM* (38.16%);

292   however, no isolates carried the *glrA*, *ermA*, or *vanA* genes. In this study, only the detection of

293   *mecA*, *ermC*, and *aacA-aphD* in the corresponding antimicrobial resistance phenotypes was

294   statistically significant (Table 5). The findings related to 11 resistance genes for seven types of

295   antibiotics are shown in Fig. 2.

296   **MLST**

297   MLST was successfully performed for all (n=76) isolates, and the results are summarized in

298   Table 6 and Fig. 3 and 4. Twenty-two STs were detected, of which ST7 accounted for 23.68%

299   (18/76), ST97 for 14.47% (11/76), ST188 for 9.21% (7/76), and ST398 for 6.57% (5/76) of the

300   isolates. The remaining STs were diverse; six novel alleles and eight new STs were detected in

301   this study, with ST7737, ST7738, ST7739, ST7740, ST7756, and ST7757 carrying new alleles

302   (Fig. 5). Although no new alleles were found for ST7735 and ST7736, new arrangements of

303   alleles existed and were also considered to be new STs. All novel alleles and STs were uploaded

304   to the PubMLST database (https://pubMLST.org/). The Simpson's diversity index of the 76

305   isolates based on MLST was 0.907, which conformed to a 95% confidence interval (0.869–

306   0.946). Clustering these STs using the eBURST algorithm revealed that ST97, ST7735, and

307   ST7756 belonged to Clonal Complex (CC)97; ST1, ST81, ST188, and ST7737 belonged to CC1;

308   ST398 belonged to CC398; ST5 and ST6 belonged to CC5; and ST2454 belonged to CC8.

309   ***spa* typing**

310   Twenty *spa* types were found among the 76 strains (Table 7). The most common type was t796

311   (23.68%, 18/76), followed by t189 (10.52%, 8/76), t359 and t091 (9.21%, 7/76 each), t034

312   (6.57%, 5/76), and t529 (5.26%, 4/76). The remaining *spa* types were found in three or fewer

313   strains and showed a scattered distribution. The Simpson's diversity index of the *spa* types of the

314   76 isolates was 0.910, and the value conformed to a 95% confidence interval (0.873–0.946).

315   **Correlation between *spa*-MLST-mapping and antimicrobial resistance**

316   ST7 was found in t796, t2383, and t091; ST97 was found in t267, t359, and t189; and ST705 was

317   found in t701 and t529. Three STs were present in t796 and two in t529, whereas the remaining

318   STs corresponded to only one *spa* type. Statistical analyses revealed a significant relevance
319   between *spa* typing and MLST (p=0.001, <0.05).
320   Oxacillin-resistant MRSA (OR-MRSA) strains mainly expressed t796-ST7 (n=7), and 93.33%
321   t796-ST7 strains were MDR. The antibiotic resistance rates of cefoxitin, clindamycin,
322   gentamicin, and temicoxin were all >70%, and those of cefoxitin, temicoxin, and cotrimoxazole
323   were significantly higher than those of other molecular types (p<0.05). The t934-CC97-ST7735
324   isolates were generally resistant to penicillin and erythromycin and 100% resistant to quinolones;
325   the resistance rate to quinolones was significantly higher than that of other molecular types
326   (p<0.05). The isolates of t796-ST7740 were OR-MRSA and resistant to penicillin, benzoxicillin,
327   and cefoxitin. In addition, other CC97 and CC398 isolates were generally resistant to penicillin,
328   but no MDR strains were detected. The detection rate of OS-MRSA was relatively high in ST97
329   (45.45%), which may be the main molecular type of OS-MRSA from dairy cows in Hunan
330   Province. The number of isolates with molecular type t189-CC1-ST188 was relatively large, but
331   the isolates showed no resistance to all drugs except for two strains that were only resistant to
332   penicillin. None of the t18636-ST7757 isolates showed resistance to any antimicrobial agent.
333   The distribution of the molecular types and specific drug resistance phenotypes of the isolates is
334   presented in Table 8.

Discussion

336   The isolation rate of *S. aureus* from raw milk in Hunan Province was 11.16%, which is lower
337   than those observed from milk sources in northern Xinjiang, China (43.1%), by Kou et al. (2021)
338   and bulk canned milk samples from Shandong Province, China (28.9%), by Zhao et al. (2021).
339   However, our results are similar to those of Bissong et al. (2020), who reported a prevalence of
340   11% for *S. aureus* in beef and milk in Cameroon. *S. aureus*, an important multi-host pathogen,
341   can be isolated from dairy cows and contaminated milk (Gurjar et al., 2012; Fetsch et al., 2014).
342   It can spread between people and livestock during grazing, activities involving human–animal
343   interactions, and movement of herds between farms (Zadoks et al., 2011). Although the isolation
344   rate of *S. aureus* from raw milk in Hunan Province is not high, these bacteria were successfully
345   isolated from the samples collected, suggesting that farmers should strictly control the cleaning
346   and disinfection of the feeding environment of dairy cows. In addition, individuals involved in
347   the dairy industry should implement standardized procedures for raw milk collection, processing,
348   and packaging and take precautionary measures to reduce environmental pollution and avoid
349   storing dairy products in unfavorable conditions to safeguard the health of consumers.
350   Serotyping of *S. aureus* CPs is usually performed to predict and determine the epidemiologic
351   trends of strains isolated in an area and the incidence of mastitis in dairy cows before molecular
352   typing (Poutrel et al., 1988). In this study, all *S. aureus* isolates exhibited either the CP5 or CP8
353   type; the overall detection rate of CP8 was higher than that of CP5, indicating that CP8 was the
354   main epidemic serotype of *S. aureus* isolated in Hunan Province. Poutrel et al. (1988) found that
355   CP5 was the main epidemic serotype in France. In a study by Han et al. (2000) in South Korea,
356   the detection rate of CP5 was similar to that of CP8, and 26% of the strains were of the CP5 type
357   and 24.2% of the CP8 type. The serotype distribution of CPs differs between countries and

358 regions, indicating that the distribution of CPs of *S. aureus* is likely related to geographical
359 location. In addition to serotyping *S. aureus*, CPs can help identify the antibody induced by its
360 protein-coupled vaccine, which has immunogenicity and has been proven to protect mice from
361 fatal *S. aureus* infection (Han et al., 2000). A targeted vaccine developed according to the
362 prevalence of CP serotypes in particular areas can yield an improved immune effect.
363 Many antibiotics are used in the animal breeding industry as therapeutics or growth enhancers
364 every year in China. However, with the evolution of bacteria and abuse of antibiotics, the
365 antibiotic resistance rate of *S. aureus* has gradually increased. It is generally believed that the
366 antibiotic resistance mechanism of *S. aureus* mainly includes the following: (1) drug hydrolase
367 and modification (such as the *aacA-aphD* gene code modifies specific groups of
368 aminoglycosides to make them lose their affinity for bacterial target ribosomes) (Wang et al.,
369 2015); (2) the active efflux system pumps drugs out of bacterial cells (for example, the
370 transporter encoded by *msr* gene can expel intracellular lincoamines to reduce intracellular drug
371 concentration); and (3) changes in the target sites of antibacterial drugs (such as *erm* gene
372 encoding methylase, which methylates the target of macrolide drug action and reduces its affinity
373 with bacterial ribosomes) (Di Modugno et al., 2002). In this study, *S. aureus* isolated from raw
374 milk from Hunan Province was highly resistant to β-lactam antibiotics, with 82.89% of the
375 isolates were resistant to penicillin. This finding is similar to that of studies from Shandong
376 (74.4%) (Zhao et al., 2021) and the Ningxia Hui Autonomous Region (94.3%) (Wang et al.,
377 2016), suggesting that *S. aureus* isolated from Chinese dairy cows is generally resistant to
378 penicillin. The mechanism of penicillin resistance in *S. aureus* is mainly through the synthesis of
379 β-lactam hydrolase, which hydrolyzes the β-lactam ring, or *mecA*-encoded PBP2a, which does
380 not easily bind with β-lactams, to replace the effect of transpeptidase PBPs, thus inhibiting the
381 ability of β-lactams to interfere with bacterial cell wall synthesis and reducing the antimicrobial
382 effect of β-lactam drugs (Hassanzadeh et al., 2020). Of the 76 isolates, 36 were found to carry
383 *mecA* and were identified as MRSA, of which 27.77% were OR-MRSA and 72.22% were OS-
384 MRSA. As Quijada et al. showed in their study (2019), deletions or mutations in the *bla* system
385 (*blaI-blaR1-blaZ*) of some isolates may downregulate the expression of *mecA*, thereby affecting
386 the resistance of the MRSA strain to oxacillin and resulting in OS-MRSA. In addition, it was
387 also found that 22.50% (9/40) of MSSA isolates were resistant to oxacillin, and *mecA*-negative
388 but oxacillin resistance-positive phenotypes have been reported in India (Kumar et al., 2010) and
389 Turkey (Turutoglu et al., 2009). This is possibly due to the role of PBP2c, which is expressed by
390 *mecC* in some strains; overexpression of β-lactamase in borderline oxacillin-resistant *S. aureus*;
391 or mutations at some amino acid sites in the PBP1, PBP2, and PBP3 transpeptidase domains,
392 which enhance the resistance of bacteria to β-lactam antibiotics, resulting in oxacillin resistance
393 despite *mec* gene negativity (Hryniewicz & Garbacz, 2017). *S. aureus* isolates were also resistant
394 to macrolides, lincosamides, aminoglycosides, florfenicol, and quinolones, which may be related
395 to the use of these drugs as the main antibiotics for treating bovine mastitis and other diseases at
396 some dairy farms. Nevertheless, there is usually no significant correlation between antibiotic
397 resistance genes and phenotypes (Bissong & Ateba, 2020) because bacterial antibiotic resistance

398 phenotypes may be determined by a variety of factors, such as environmental changes, strain
399 activity, and frequent use of antimicrobial agents, whereas genotypes are decided only by
400 internal gene factors. In this study, a strain of MRSA was also found to be resistant to
401 vancomycin. The strain was identified as VRSA, but *vanA* was not detected. Bhattacharyya et al.
402 (2016) found that the emergence of VRSA was not completely related to *vanA* positivity, and
403 Roch et al. (2014) proved that *S. aureus* could trigger the vancomycin-resistant phenotype
404 through exposure to non-glycopeptide antibiotics, such as β-lactams. The development of
405 vancomycin resistance in *S. aureus* could also be related to epigenetic processes and not only to
406 gene expression (Haaber et al., 2015), which could explain why the only strain of VRSA in this
407 study was also an MDR OR-MRSA.
408 A large international database containing the sequence data of thousands of *S. aureus* strains has
409 been established. It is an important data source for large-scale epidemiological and genetic
410 correlation research on *S. aureus* (van Wamel et al., 2010). In this study, we found abundant and
411 diverse STs of *S. aureus* isolated from raw milk in Hunan Province, with ST7 being the most
412 common. Some studies have shown that ST398 is the main ST related to livestock infections,
413 and ST5, 45, and 239 are related to hospital-acquired infections. ST1, 8, 80, and 59 are mainly
414 associated with community-acquired infection (Skov & Jensen, 2009), while ST97 is commonly
415 associated with *S. aureus*-induced mastitis worldwide (Smith et al., 2005). In this study, CC398-
416 ST398, CC5-ST5, CC1-ST1, and CC97-ST97 were all detected, which further confirmed the
417 threat posed by raw milk infected with *S. aureus* to the dairy industry and public health. It is
418 inferred that pathogenic bacteria may spread to the environment and infect humans during
419 milking and transportation and via inadequately sterilized dairy products. Additionally, a
420 previous study reported that alleles having only a single nucleotide mutation should be labeled as
421 a novel ST (Enright et al., 2000). In this study, CC1-ST7737, ST7738, ST7739, ST7740, CC97-
422 ST7756, and ST7757 showed altered nucleotide sequences, but only CC97-ST7735 and ST7736
423 showed a new allele arrangement. The emergence of these new STs is not accidental, and related
424 mutants might have appeared a long time ago and spread among dairy cows in Hunan Province.
425 However, these new STs were only discovered recently, possibly due to the lack of research on
426 the molecular typing of *S. aureus* in raw milk from Hunan. The detection rate of MRSA in the 17
427 strains with newly discovered STs was 52.94%, of which ST7738 and 7740 were MRSA,
428 indicating that these new STs may become an important molecular type of MRSA in Hunan
429 Province. Furthermore, the only VRSA strain exhibited ST7739, indicating that the monitoring
430 of new STs from isolates obtained during epidemics using MLST should be strengthened.
431 Twenty *S. aureus spa* types were identified in this study. Diversity analysis showed that the
432 diversity of *spa* typing was high; t796 was the most common, followed by t189, t359, and t091.
433 Mekonnen et al. (2018) identified 20 *spa* types from 79 strains of *S. aureus* isolated from milk
434 samples from dairy farms in northwest Ethiopia, and t042 was the main *spa* type. Strommenger
435 et al. (2006) studied *S. aureus* isolated from Dutch cows with mastitis and found that t529 was
436 the dominant *spa* type. Upon comparing the above results, we speculate that isolates from
437 different countries and regions usually have different molecular types. Many studies have used

438   MLST and *spa* typing to determine the population structure of highly cloned *S. aureus*
439   (Bhargava et al., 2011; Basanisi et al., 2017). Malachowa et al. (2005) used pulse field gel
440   electrophoresis, MLST, multiple locus variable-number tandem repeat analysis, and *spa* typing
441   to compare 59 strains of iatrogenic *S. aureus* and found that *spa* typing showed similar results to
442   those of MLST. They suggested that multiple typing methods should be integrated to improve
443   the accuracy of *S. aureus* typing. In this study, there was a significant correlation between *spa*
444   typing and MLST. Although *spa* types and STs with multiple corresponding genotypes were
445   observed, most STs and *spa* types corresponded to 1 genotype. The existence of different STs
446   associated with the same *spa* type indicates that 2 STs may have originated from the same clone
447   and evolved in parallel (Strommenger et al., 2008). The *spa*-MLST-mapping typing of the 76
448   isolates clearly reflected the molecular characteristics of *S. aureus* isolates in Hunan Province
449   and that t796-ST7 was the main molecular type of *S. aureus*. Notably, five isolates exhibited
450   t034-CC398-ST398, which has been reported to be the main molecular type of *S. aureus*
451   infection in livestock and poultry (Bouiller et al., 2016), and MRSA isolates exhibiting t034-
452   CC398-ST398 have been discovered in domestic pigs (Graveland et al., 2011). It is speculated
453   that t034-CC398-ST398 isolates may spread among different kinds of livestock in different
454   regions, and they may even spread between livestock and humans and cause diseases (van Cleef
455   et al., 2011); therefore, farmers should be vigilant.
456   Through the isolation and identification of milk-derived *S. aureus* in northern Italy, Gazzola et
457   al. (2020) found that CC398, CC1, and CC97 were three common MRSA clones, and Li et al.
458   (2015) found that the main molecular type of MRSA isolated from milk in Shaanxi Province was
459   t524-ST71. These studies suggest that the distribution of MRSA may be related to its molecular
460   typing. In this study, different molecular isolates showed different antibiotic resistance
461   characteristics, and the most common molecular type exhibited by MRSA was t796-ST7. The
462   isolates of the same molecular type may have the same or similar antibiotic resistance spectrum.
463   The isolates obtained from different regions or sources may have different molecular types, and
464   the isolates of different molecular types may have different antibiotic resistance, which is
465   speculated to result from the competition between different molecular types under the screening
466   pressure of antibiotics in different regions, and the prevalence of dominant molecular types can
467   promote further spread of antibiotic-resistant bacteria. In addition, Enright et al. (2000) pointed
468   out that it is usually impossible for unrelated MRSA and MSSA to have the same molecular
469   typing and that this status may be caused by the horizontal transfer of *mecA* from MRSA to
470   MSSA or the loss or inactivation of the *mec* gene of some MRSA strains, resulting in some
471   MRSA strains acquiring the same allele map as their MSSA ancestors and thus being classified
472   as having the same STs.

473   **Conclusions**
474   The results showed that *S. aureus* from raw milk in Hunan area had a typical CP type, rich
475   molecular typing, and different levels of antibiotic resistance to a variety of antibiotics. Our
476   findings could help promote the production of *S. aureus* vaccine against CP8 in Hunan area and
477   resistance monitoring of t796-ST7 isolates with the prevalent molecular type of MDR strains. A

478 The present results suggest reducing the use of β-lactam, macrolide, lincoamine antibacterial
479 drugs and appropriately administering doxycycline, sulfonamides, and glycopeptides as
480 veterinary antibiotics to treat infectious diseases in dairy cows. The antibiotic drug dosage should
481 be strictly controlled, and drug rotation and the withdrawal period of the antibiotics should be
482 strictly implemented. Further, active research, development, and promotion of Chinese herbal
483 medicines, micro-ecological preparations, and other new non-antibiotic drugs are necessary.

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740

741

Figure 1

Capsular polysaccharide detection

All isolates were classified into capsular polysaccharide serotypes 5 and 8 (CP5 and CP8).

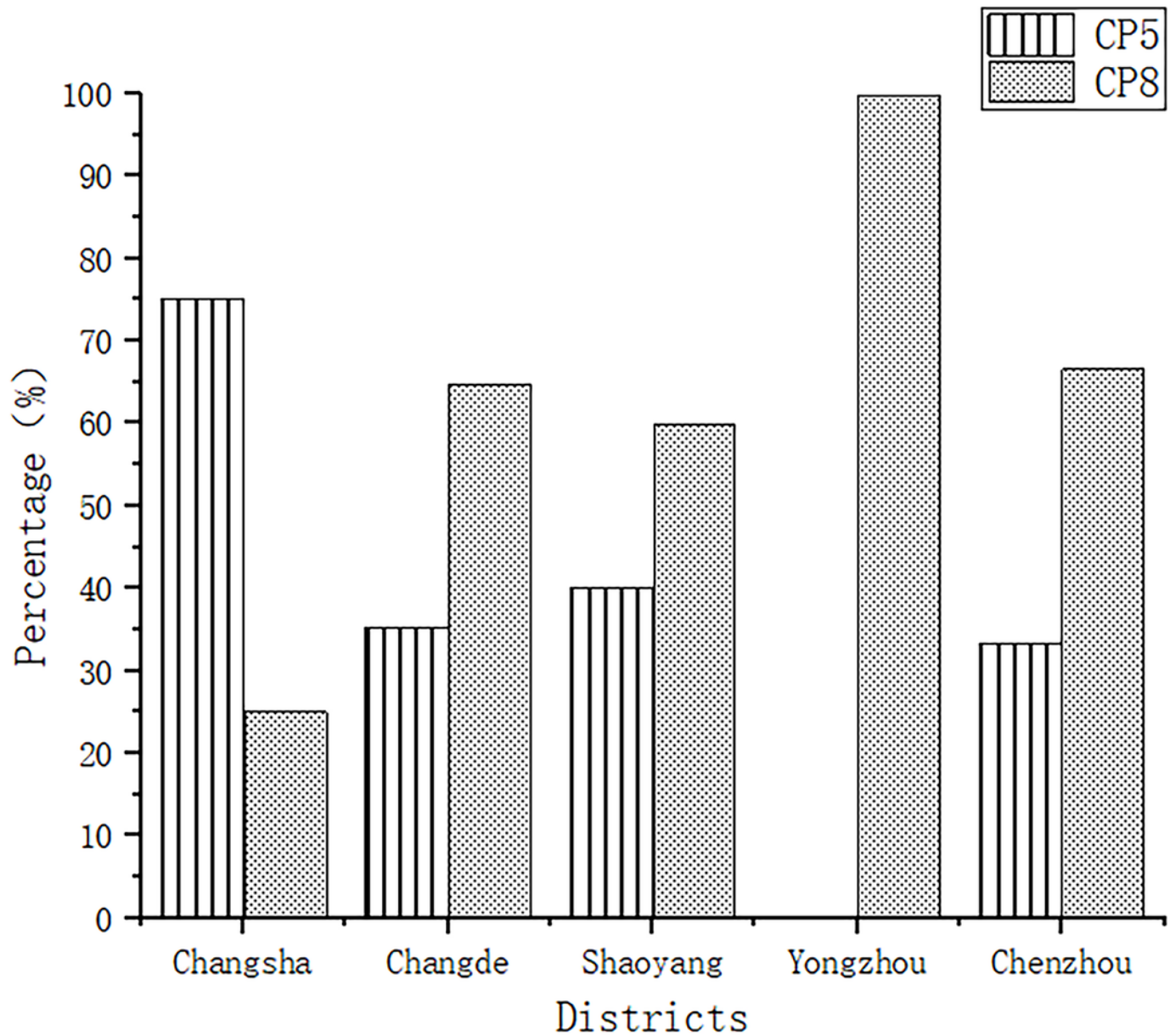



Figure 2

Detection rate of antimicrobial resistance genes

Figure showing bar chart of resistance rate (%) for antibiotic resistance genes

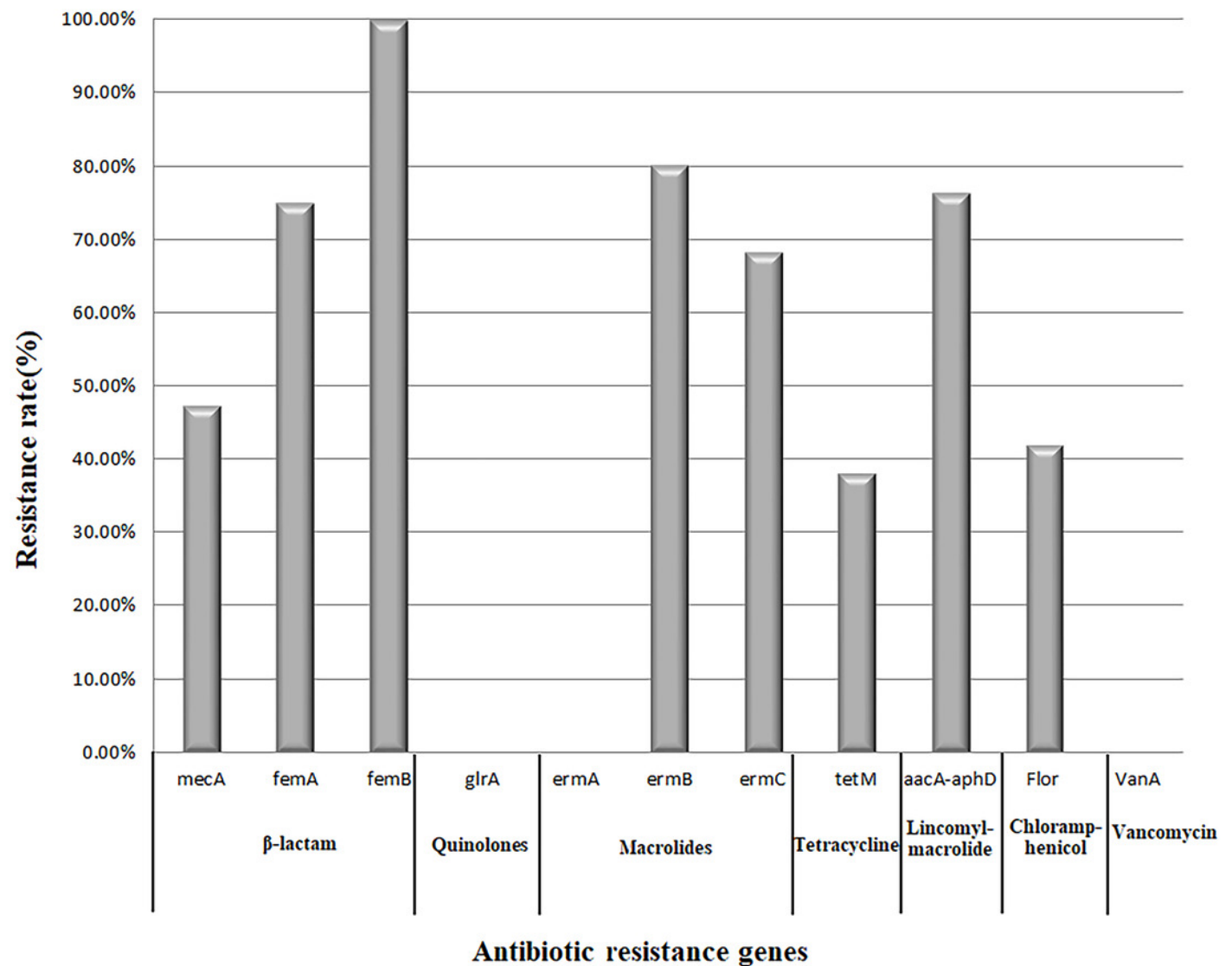



Figure 3

Distance matrix showing genetic relationship between classes of sequence typing (ST)

Each horizontal row and column represent a class of sequence typing (ST). The color of each coordinate indicates the distance of the genetic relationship between two STs. The closer the genetic relationship is the smaller the allele difference is, and the smaller the corresponding distance difference is. The ST difference in the same clonal complex group is less than 2.00.

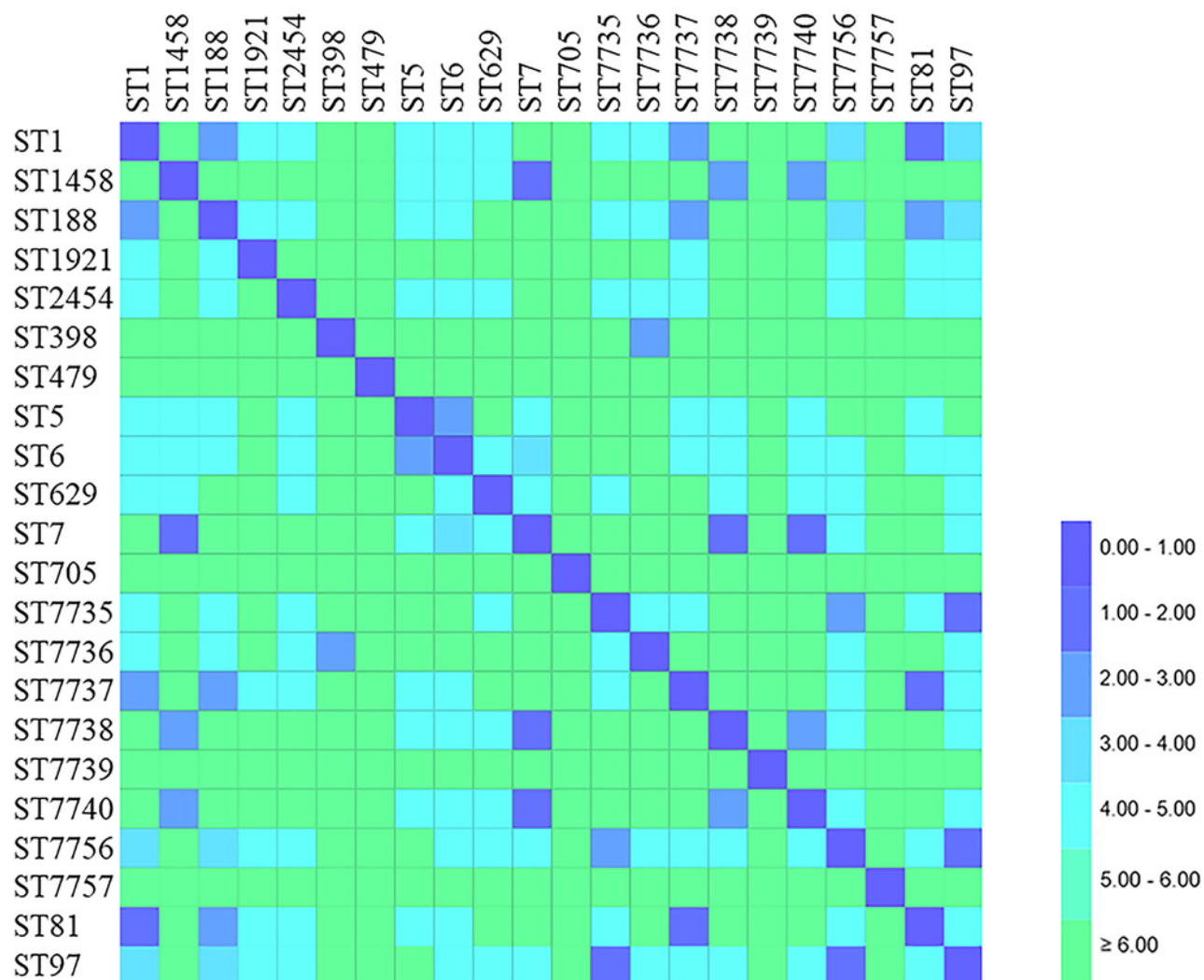



Figure 4

Minimum Spanning Tree (MST)

The nodes of different colors represent different MLST types, and the size represents the relative number of isolates. The values between the lines represent the number of different alleles between two adjacent STs, and nodes with allelic differences of more than 4 are connected by dotted lines. The STs from common CCs had at least five same alleles.

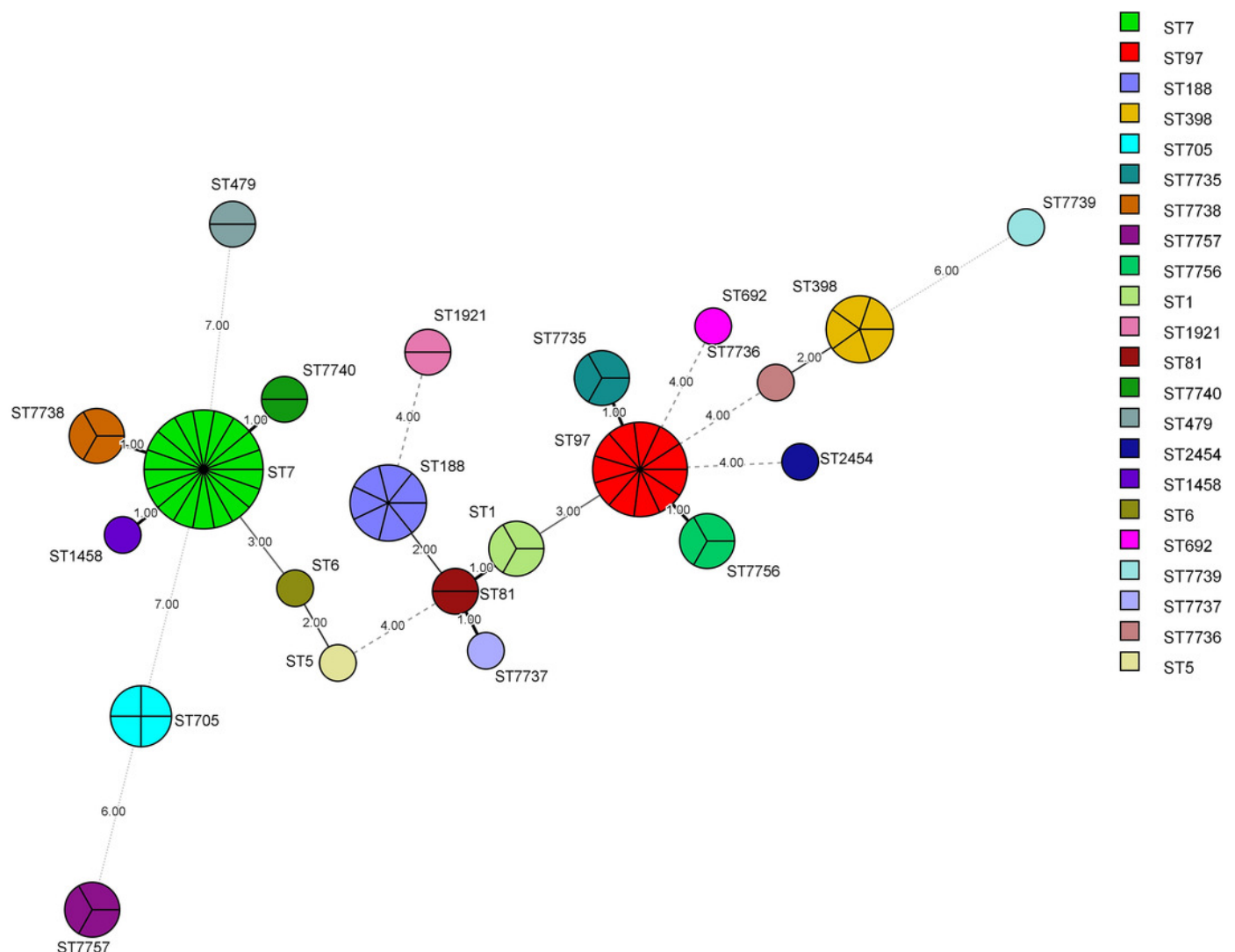
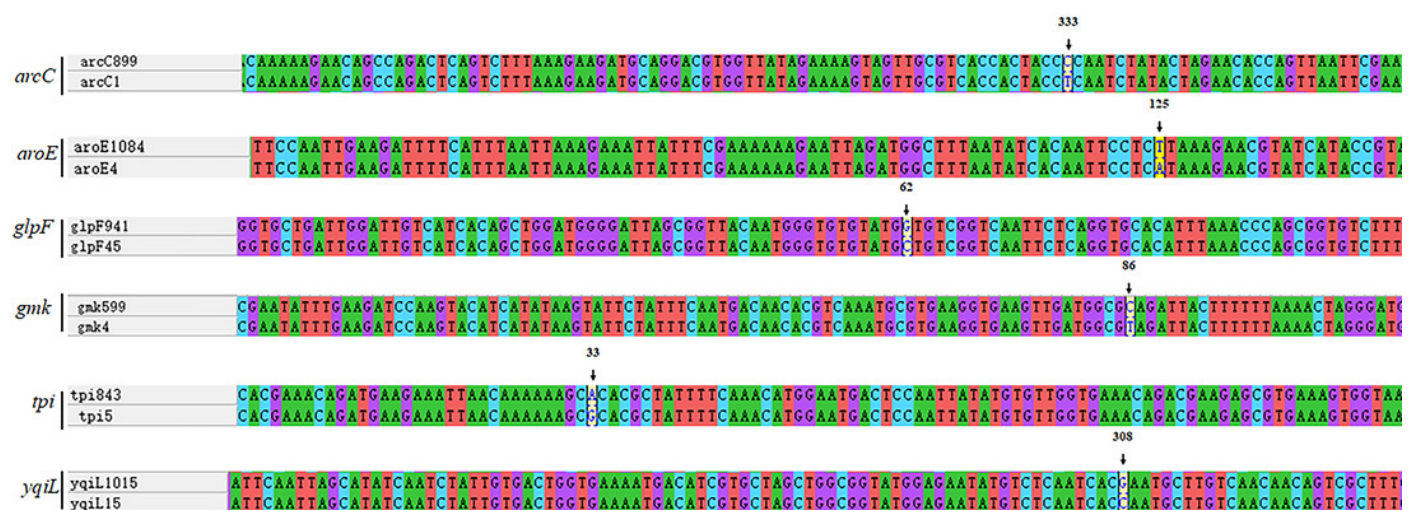



Figure 5

Analysis of nucleic acid sequence of novel alleles

Every new allele was compared to its closest matches. ST7737 *arcC*:333T→C; ST7738 *aroE*:125A→T; ST7739 *glpF*:62C→G; ST7740 *gmk*:86T→C; ST7756 *tpi*:33G→A; ST7757 *yqiL*:308C→G.

Sequences of primer sets with their corresponding polymerase chain reaction protocols and product sizes

1 **TABLE 1** Sequences of primer sets with their corresponding polymerase chain reaction
2 protocols and product sizes

Target gene	Primer sequence(5′—3′)	Amplicon size(bp)	Annealing temperature(℃)
nuc	GCGATTGATGGTGATACGGTTAAAT AACATAAGCAACTTTAGCCAAGCC	294	56
cp5	GTCAAAGATTATGTGATGCTACTGA G ACTTCGAATATAAACTTGAATCAAT GTTATACAG	361	62
cp8	GCCTTATGTTAGGTGATAAACC GGAAAAACACTATCATAGCAGG	173	62
mecA	TGGCTCAGGTACTGCTATCC CACCTTGTCCGTAACCTGAA	533	56
femA	TGCTGGTAATGATTGGTT ATCTCGCTTGTTATGTGC	498	56
femB	TTACAGAGTTAACTGTTACC ATACAAATCCAGCACGCTCT	651	55
ermA	TCTAAAAAGCATGTAAAAGAA CTTCGATAGTTTATTAATATTAG	645	52
ermB	GAAAAGGTACTCAACCAAATA AGTAACGGTACTTAAATTGTTTAC	639	45
ermC	GCTAATATTGTTTAAATCGTCAATTC C	572	50
tetM	GGATCAGGAAAAGGACATTTTAC	158	55
aacA-aphD	AGTGGAGCGATTACAGAA CATATGTCCTGGCGTGTCTA	220	56

glrA	CCAAGAGCAATAAGGGCATA CACTATCATAACCACTACCG	457	55
VanA	AGGGAAGTGTTTCAGTCT CCATTCTCAATAATACCG	885	55
Flor	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	900	55
arcC	TGCCAGCAGTGCCGTTTAT CACCGCCCAAGCAGAAGTA	456	55
aroE	TTGATTCACCAGCGCGTATTGTC AGGTATCTGCTTCAATCAGCG	456	55
glpF	ATCGGAAATCCTATTTCACATTC GGTGTTGTATTAATAACGATATC	465	55
gmk	CTAGGAACTGCAATCTTAATCC TGGTAAAATCGCATGTCCAATTC	429	55
pta	ATCGTTTTATCGGGACCATC TCATTAACTACAACGTAATCGTA	474	55
tpi	GTTAAAATCGTATTACCTGAAGG GACCCTTTTGTTGAAAAGCTTAA	402	55
yqiL	TCGTTCATTCTGAACGTCGTGAA TTTGCACCTTCTAACAATTGTAC	516	55
spa	CAGCATACAGGACACCTATTGGC CGTTGAGGAATCGATACTGGAAC	Variation	58
	TAAAGACGATCCTTCGGTGAGC CAGCAGTAGTGCCGTTTGCTT		

Results of VITEK 2 automatic microbial biochemical identification

1 **TABLE 2 Results of VITEK 2 automatic microbial biochemical identification**
2

Details of biochemical reaction											
AMY	-	PIPLC	-	dXYl	-	ADH1	+	BGAL	-	AGLU	+
APPA	-	CDEX	-	AspA	-	BGAR	-	AMAN	-	PHOS	+
LeuA	-	ProA	-	BGURr	-	AGAL	-	PyrA	+	BGUR	-
AlaA	-	TyrA	-	dSOR	-	URE	-	POLYB	+	dGAL	+
dRIB	-	ILATk	+	LAC	-	NAG	-	dMAL	+	BACI	+
NOVO	-	NC6.5	+	dMAN	+	dMNE	-	MBdG	-	PUL	-
dRAF	-	O129R	+	SAL	-	SAC	-	dTRE	+	ADH2s	-
OPTO	+										

4 AMY(Amygdalin); PIPLC(Phosphatidyl phospholipase C); dXYl(D-xylose); ADH1(Arginine
5 dihydrolase 1); BGAL(β-D-galactosidase); AGLU(α-glucosidase); APPA(Alanine-phenylalani
6 ne-proline arylaminase); CDEX(Cyclodextrin); AspA(L-aspartate arylaminase); BGAR(β-gal
7 actose pyranosidase); AMAN(α-mannosidase); PHOS(Phosphatase); LeuA(Leucine arylamin
8 ase); ProA(L-proline arylaminase); BGURr(β-glucosidase); AGAL(α-galactosidase); PyrA(P
9 yroglutamate arylaminase); BGUR(β-D-glucosidase); AlaA(Alanine arylaminase); TyrA(Tyr
10 osine arylaminase); dSOR(D-sorbitol); URE(Urease); POLYB(Polymyxin B tolerance); dG
11 AL(D-galactose); dRIB(D-ribose); ILATk(L-Lactate produces alkali); LAC(Lactose); NAG(
12 N-acetyl-D-glucosamine); dMAL(D-maltose); BACI(Bacitracin tolerance); NOVO(Neomycin
13 tolerance); NC6.5(6.5% sodium chloride growth); dMAN(D-mannitol); dMNE(D-Mannose);
14 MBDG(Methyl-B-D-glucopyranoside); PUL(Pullulan); dRAF(D-raffinose); O129R(O/129 tol
15 erance); SAL(Salicin); SAC(Sucrose); dTRE(D-trehalose); ADH2s(Arginine dihydrolase 2);
16 OPTO(Optokhin tolerance)
17
18 +, Reaction positive; -, Reaction negative
19
20

Antibiotics resistance of *Staphylococcus aureus* isolates

The breakpoint of antimicrobial resistance was judged according to the standard of the Clinical and Laboratory Standards Institute. The isolates resistant to oxacillin and vancomycin were verified by three repeated tests. OS-MRSA, oxacillin-sensitive mecA-positive *S. aureus*; OR-MRSA, oxacillin-resistant mecA-positive *S. aureus*

1 **TABLE 3** Antibiotics resistance of *Staphylococcus aureus* isolates

Antibiotics		Resistance MIC (μg·mL⁻¹)	No(%) of resistant isolates(n=76)			Total
			OS-MRSA	OR-MRSA	MSSA	
β-lactams	Penicillin	≥0.25	19(25.00%)	10(13.16%)	34(44.74%)	63(82.89%)
	Cefoxitin	≥8	2(2.63%)	9(11.84%)	6(7.89%)	17(22.37%)
	Oxacillin	≥4	0(0.00%)	10(13.16%)	9(11.84%)	19(25.00%)
	Ceftiofur	≥8	0(0.00%)	0(0.00%)	2(2.63%)	2(2.63%)
	Augmentin	≥8	1(1.32%)	1(1.32%)	1(1.32%)	3(3.95%)
Macrolides	Erythromycin	≥8	4(5.26%)	5(6.58%)	8(10.53%)	17(22.37%)
	Tilmicosin	≥32	2(2.63%)	8(10.53%)	6(7.89%)	16(21.05%)
Lincosamides	Clindamycin	≥4	2(2.63%)	8(10.53%)	7(9.21%)	17(22.37%)
Quinolones	Ofloxacin	≥4	1(1.32%)	0(0.00%)	4(5.26%)	5(6.58%)
	Enrofloxacin	≥4	1(1.32%)	0(0.00%)	2(2.63%)	3(3.95%)
Tetracyclines	Doxycycline	≥16	0(0.00%)	0(0.00%)	0(0.00%)	0(0.00%)
Aminoglycosides	Gentamicin	≥32	2(2.63%)	8(10.52%)	5(6.58%)	15(19.73%)
Sulfonamides	Sulfisoxazole	≥512	0(0.00%)	2(2.63%)	1(1.32%)	3(3.95%)
	Trimethoprim S-ulfamethoxazole	≥4	0(0.00%)	4(5.26%)	4(5.26%)	8(10.53%)
Phenicols	Florfenicol	≥8	1(1.32%)	0(0.00%)	5(6.58%)	6(7.89%)
Glycopeptides	Vancomycin	≥16	0(0.00%)	1(1.32%)	0(0.00%)	1(1.32%)
Diterpenes	Tiamulin	≥32	0(0.00%)	1(1.32%)	1(1.32%)	2(2.63%)
Oxazolidinones	Linezolid	≥8	0(0.00%)	1(1.32%)	1(1.32%)	2(2.63%)

2 The breakpoint of antimicrobial resistance was judged according to the standard of the Clinical
3 and Laboratory Standards Institute. The isolates resistant to oxacillin and vancomycin were
4 verified by three repeated tests.

5 OS-MRSA, oxacillin-sensitive mecA-positive *S. aureus*; OR-MRSA, oxacillin-resistant mecA-
6 positive *S. aureus*

Spectrum of multi-drug resistance.

1   **TABLE 4** Spectrum of multi-drug resistance.

Type of antimicrobial resistance	Number of strains(%)	Strain number	Antibiotic resistance spectrum
macrolides-β-lactam-quinolones-glycopeptides-diterpenoids-oxazolidinones	1(4.76%)	SY-14	PEN-ERY-OXA-VAN-TIA-TIL-LZD
macrolides-β-lactam-sulfonamides-aminoglycosides-lincosamides	8(38.09%)	CD-15	PEN-ERY-OXA-CLI-SF-SXT-TIL-GEN
		CD-16	PEN-OXA-CLI-CEF-CFX-OXA-SXT-TIL-GEN
		CD-21	PEN-OXA-CLI-CEF-CFX-OXA-SXT-TIL-GEN
		CD-22	PEN--CLI -CFX-SF-OXA-SXT-TIL-GEN
		CD-23	PEN--CLI -CFX-SF-OXA-SXT-TIL-GEN
		CD-24	PEN-OXA-CLI-CFX-SF-OXA-SXT-TIL-GEN
		CD-25	PEN-CFX-CLI-OXA-SXT-TIL-GEN
		CD-26	PEN--CLI -CFX-SF-OXA-SXT-TIL-GEN
macrolides-β-lactam-aminoglycosides-lincosamides	5(23.80%)	CD-1	PEN-ERY-CLI-CFX-TIL-GEN
		CD-2	PEN-ERY-CFX-CLI-OXA-TIL-GEN
		CD-5	PEN-ERY-CFX-CLI-OXA-TIL-GEN
		CD-6	CFX-CLI-ERY-A/C-PEN-OXA-TIL-GEN
		CD-7	PEN-ERY-CFX-CLI-A/C-TIL-GEN
macrolides-β-lactam-lincosamides-aminoglycosides	2(9.52%)	CD-34	PEN-ERY-CLI-CFX-OXA-GEN
		SY-8	PEN-CFX-CLI-ERY-OXA-TIL-GEN
quinolones-macrolides-β-lactam-sulfonamides	1(4.76%)	CS-3	PEN-ERY-OFL-ENR-SF
quinolones-macrolides-β-lactam	2(9.52%)	CS-1	PEN-ERY-OFL-EN
		CS-2	PEN-ERY-OFL-EN
macrolides-β-lactam-lincosamides	1(4.76%)	CD-8	PEN-ERY-CLI
chloramphenicol-diterpenoids-oxazolidinones	1(4.76%)	YZ-2	FFC-TIA-LZD

Comparison of genotypic and phenotypic characteristics for antimicrobial resistance in *Staphylococcus aureus* isolates.

The phenotypes of β-lactam resistance were based on penicillin, oxacillin, cefoxitin, ceftiofur, and augmentin, while those of macrolide-lincomide resistance were based on erythromycin, tilmicosin, and clindamycin; tetracycline resistance phenotype was based on doxycycline; aminoglycoside resistance phenotype was based on gentamicin; and chloramphenicol resistance phenotype was based on florfenicol. The association between resistance phenotype and resistance genes was significant (p<0.05). G+, resistance gene positive; P+, phenotypic resistance

1 **TABLE 5** Comparison of genotypic and phenotypic characteristics for antimicrobial resistance in *Staphylococcus aureus* isolates.

Antibiotics	Genes	Characteristics of *S. aureus* isolates		Association p
		G$^+$ No(%)	P$^+$ No(%)	
β-lactams	*mecA*	36(47.37%)	63(82.89%)	0.0002
	femA	57(75.00%)		0.861
Macrolides-Lincoamines	*ermB*	61(80.26%)	25(32.89%)	0.229
	ermC	52(68.42%)		0.011
Tetracyclines	*tetM*	29(38.16%)	0(0.00%)	1.000
Aminoglycosides	*aacA-aphD*	58(76.32%)	15(19.73%)	0.017
Phenicols	*Flor*	32(42.11%)	6(7.89%)	0.685

2 The phenotypes of β-lactam resistance were based on penicillin, oxacillin, cefoxitin, ceftiofur, and augmentin, while those of macrolide-lincomide

3 resistance were based on erythromycin, tilmicosin, and clindamycin; tetracycline resistance phenotype was based on doxycycline; aminoglycoside

4 resistance phenotype was based on gentamicin; and chloramphenicol resistance phenotype was based on florfenicol.

5 The association between resistance phenotype and resistance genes was significant ($p<0.05$).

6 G+, resistance gene positive; P+, phenotypic resistance

MLST distribution of *Staphylococcus aureus* isolates

*Novel ST types. †Novel alleles. CI, confidence interval; MLST, multi-locus sequence typing; ST, sequence typing.

1   **TABLE 6** MLST distribution of *Staphylococcus aureus* isolates

STs[a]	clonal complex	No(%) of isolates	Allelic profiles[b]							Simpson's diversity index (95% CI[c])
			arcC	aroE	glpF	gmk	pta	tpi	yqiL	
ST7		18(23.68%)	5	4	1	4	4	6	3	
ST97	97	11(14.47%)	3	1	1	1	1	5	3	
ST188	1	7(9.21%)	3	1	1	8	1	1	1	
ST398	398	5(6.57%)	3	35	19	2	20	26	39	
ST705		4(5.26%)	6	72	50	43	49	67	59	
ST7735*	97	3(3.94%)	3	1	605	1	1	5	3	
ST7738*		3(3.94%)	5	1084†	1	4	4	6	3	
ST7756*	97	3(3.94%)	3	1	1	1	1	843†	3	
ST7757*		3(3.94%)	6	125	213	27	13	13	1015†	
ST1	1	3(3.94%)	1	1	1	1	1	1	1	
ST81	1	2(2.63%)	1	1	1	9	1	1	1	0.907(0.869-0.946)
ST479		2(2.63%)	52	79	54	18	56	32	65	
ST1921		2(2.63%)	4	9	1	8	1	10	218	
ST7740*		2(2.63%)	5	4	1	599†	4	6	3	
ST5	5	1(1.31%)	1	4	1	4	12	1	10	
ST6	5	1(1.31%)	12	4	1	4	12	1	3	
ST629		1(1.31%)	12	89	1	1	4	5	90	
ST1458		1(1.31%)	5	4	1	4	4	6	39	
ST2454	8	1(1.31%)	3	3	1	1	264	1	10	
ST7736*		1(1.31%)	3	35	19	1	1	26	39	
ST7737*	1	1(1.31%)	899†	1	1	9	1	1	1	
ST7739*		1(1.31%)	18	95	941†	2	7	15	5	

2   *Novel ST types. †Novel alleles.

3   CI, confidence interval; MLST, multi-locus sequence typing; ST, sequence typing.

Spa types distribution of *Staphylococcus aureus* isolates

1   **TABLE 7** *Spa* types distribution of *Staphylococcus aureus* isolates

Spa types	No.of isolates	Repeat succession	Simpson's diversity index(95% CI)
t796	18(23.68%)	07-23-21-17-12-23-02-12-23	
t189	8(10.52%)	07-23-12-21-17-34	
t359	7(9.21%)	07-23-12-21-17-34-34-33-34	
t091	7(9.21%0	07-23-21-17-34-12-23-02-12-23	
t034	5(6.57%)	08-16-02-25-02-25-34-24-25	
t529	4(5.26%)	04-34	
t267	3(3.94%)	07-23-12-21-17-34-34-34-33-34	
t2383	3(3.94%)	08-16	
t18636	3(3.94%)	121-21-16-34-17-82-24-17-17	
t114	3(3.94%)	07-16-34-33-13	
t934	3(3.94%)	07-23-12-34-34-34-34-33-34	0.910(0.873-0.946)
t543	2(2.63%)	04-20-17	
t5100	2(2.63%)	07-21-16-34-33-13	
t164	2(2.63%)	07-06-17-21-34-34-22-34	
t7295	1(1.31%)	04-31-17-17	
t701	1(1.31%)	11-10-21-17-34-24-34-22-25-25	
t2700	1(1.31%)	07-16-12-23-02-12-23-02-02-34	
t2247	1(1.31%)	26-25-17	
t19817	1(1.31%)	07-21-16-34	
t002	1(1.31%)	26-23-17-34-17-20-17-12-17-16	

1   **TABLE 8** Analysis of *spa*-MLST-mapping and MRSA

Spa typing	STs(CCs)	No.of resistant isolates(%)		
		OS-MRSA[b]	OR-MRSA[c]	MSSA
t934	CC97-ST7735	1(1.31%)	0(0.00%)	2(2.63%)
t796	ST7	2(2.63%)	7(9.21%)	6(7.89%)
	ST7756	1(1.31%)	0(0.00%)	0(0.00%)
	ST7740	0(0.00%)	2(2.63%)	0(0.00%)
t7295	ST7739	0(0.00%)	1(1.31%)	0(0.00%)
t701	ST705	0(0.00%)	0(0.00%)	1(1.31%)
t543	ST479	1(1.31%)	0(0.00%)	1(1.31%)
t529	ST705	1(1.31%)	0(0.00%)	2(2.63%)
	CC5-ST6	1(1.31%)	0(0.00%)	0(0.00%)
t5100	CC1-ST81	0(0.00%)	0(0.00%)	2(2.63%)
t359	CC97-ST97	2(2.63%)	0(0.00%)	5(6.57%)
t2700	CC8-ST2454	0(0.00%)	0(0.00%)	1(1.31%)
t267	CC97-ST97	2(2.63%)	0(0.00%)	1(1.31%)
t2383	ST7	0(0.00%)	0(0.00%)	1(1.31%)
	ST1458	0(0.00%)	0(0.00%)	1(1.31%)
	ST7736	1(1.31%)	0(0.00%)	0(0.00%)
t2247	ST692	1(1.31%)	0(0.00%)	0(0.00%)
t19817	CC1-ST7737	0(0.00%)	0(0.00%)	1(1.31%)
t189	CC1-ST188	3(3.94%)	0(0.00%)	4(5.26%)
	CC97-ST97	1(1.31%)	0(0.00%)	0(0.00%)
t18636	ST7757	3(3.94%)	0(0.00%)	0(0.00%)

t164	ST1921	1(1.31%)	0(0.00%)	1(1.31%)
t114	CC1-ST1	3(3.94%)	0(0.00%)	0(0.00%)
	ST7	0(0.00%)	0(0.00%)	2(2.63%)
t091	ST7738	0(0.00%)	0(0.00%)	3(3.94%)
	CC97-ST7756	0(0.00%)	0(0.00%)	2(2.63%)
t034	CC398-ST398	2(2.63%)	0(0.00%)	3(3.94%)
t002	ST5	0(0.00%)	0(0.00%)	1(1.31%)

3   MLST, multi-locus sequence typing; MRSA, methicillin-resistant Staphylococcus aureus