Prevalence and characterization of Staphylococcus aureus isolated from pasteurized milk in China

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ABSTRACT

Staphylococcus aureus is one of the most important food-borne pathogens globally. It produces various toxins and invasive enzymes and can be found in numerous food products. Milk is an important source of staphylococcal food poisoning. After pasteurization, this microorganism or its enterotoxins might still remain in pasteurized milk. Therefore, this study was to investigate the contamination of *S. aureus* in 258 pasteurized milk from 39 cities of China. The prevalence and levels of *S. aureus* in these samples as well as antibiotic susceptibility profiles, virulence genes, biofilm formation and biofilm related genes, *spa* typing and MLST were used to determine the characterization among the isolates. It was found 3.9% of samples were detected *S. aureus* in 8 of 39 cities in China. The contaminated level were not very excessive which showed the MPN values of the most positive samples (9/10) were less than 1 MPN/g. All pasteurized milk-related *S. aureus* isolates have ability to produce biofilm and harbored *icaA, icaD, eno*, *clfA, clfB, fnbA, fnbB, fib* genes, other biofilm related genes *icaC* were showed in 91.7% of isolates and *cna* gene were showed in 50%, except *bap* gene which were free in all isolates. The antibiotic susceptibility test showed that all isolates were resistant or intermediate-resistant to different concentrations of the antibiotics. Furthermore, 75.0% of the isolates were resistant to three or more antibiotic classes, which indicated multidrug resistance. The isolates had virulence potential, which showed 66.7% (8/12) of the isolates carried one or more virulence-associated genes. Molecular typing by MLST and *spa* typing enabled classification of these isolates into a total of 11 sequence types (STs) and *spa* types, which indicated high genetic diversity. Most of these types were related to various clinical *S. aureus* infections. Thus, the findings of this study reflect the potential risk of *S. aureus* infection in China. Our study also provides comprehensive analysis of the prevalence of *S. aureus* in pasteurized milk and helps ensure more accurate treatment of human infection with effective antibiotics.

Keywords: *Staphylococcus aureus*; Pasteurized milk; Risk assessment; Virulence genes; Antibiotic resistance; Biofilm formation; MLST; *spa* typing

1. Introduction

*Staphylococcus aureu*s is a pathogen associated with serious community and hospital-acquired diseases. It has low nutritional requirements and widely exists in nature. In China, microbial food poisoning accounted for 53.7% of food poisoning emergencies in 2015. Of which, *S. aureus* was an important pathogenic factor in these cases (Wu et al., 2018a). In the United States, *S. aureus* causes approximately 241,000 cases of food poisoning each year (Scallan et al., 2011; Kadariya et al., 2014). This foodborne pathogen is considered as one of the world's leading causes of disease outbreaks related to food consumption, being responsible for a variety of manifestations and diseases (Jamali et al., 2015).

S. aureus produces a variety of toxins and invasive enzymes such as staphylococcal enterotoxins (SEs), hemolysins, Panton-Valentine leukocidin (PVL), toxic shock syndrome toxin-1 (TSST-1), plasma coagulase, and deoxyribonuclease (Spanu et al., 2012). Differentiation between virulent and nonvirulent strains is significant for evaluating the potential implications of the presence of this microorganism for food safety and public health. In which, SEs are active at concentrations ranging from high nanogram to low microgram quantities (Larkin et al., 2009) and are resistant to conditions (heat treatment, low pH) that retaining their activity in the digestive tract after ingestion (Evenson et al., 1988; Argud ń et al., 2010). In addition, TSST-1 is a superantigenic exotoxin that causes toxic shock syndrome and PVL is a bacteriophage-encoded bicomponent leukotoxin that is in some strains of *S. aureus* and plays a key role in leukocytolysis and tissue necrosis (Shallcross et al., 2013). *S. aureus* often develops antibiotic resistance. Isolation of single- or multiple-drug resistant *S. aureus* (MRSA) strains from food, the environment, and clinics has been constantly reported (Gould et al., 2012; Rasigade et al., 2014). The ability of *S. aureus* to form biofilms helps the bacterium to survive in hostile environments within the host and is considered to be responsible for chronic or persistent infections (Costerton et al., 1999). The ability of some strains to synthesize biofilms could increase their pathogenicity since established biofilms can tolerate antimicrobial agents, thus making the bacterium extremely difficult to eradicate (Zmantar et al., 2010).

Nowadays, molecular typing methods are crucial in epidemiological investigations of food processing and enhance the resolution of surveillance (Shi et al., 2015). Of various subtyping approaches, multilocus sequence typing (MLST) is a widely accepted method of DNA

sequence based typing that based on analysis of relatively conserved genes that encode essential proteins. For *S. aureus*, the level of discrimination provided by MLST is sufficient to provide a relatively detailed picture of the global dissemination of the organism (Saunders and Holmes, 2007). *spa* typing is another efficient typing method for *S. aureus*, it based on sequencing of the polymorphic X region of the protein A gene (*spa*) (Hallin et al., 2009). Similar to MLST, it suggests that *spa* typing is suitable for epidemiology and evolutionary investigations based on studies of European and international isolates (Wu et al., 2018b).

Milk is an important source of staphylococcal food poisoning. There are several foodborne outbreaks of *S. aureus* intoxications have been documented to be associated with consumption of contaminated milk (De Buyser et al., 1985; Miwa etal., 2001; Fetsch et al., 2014). In addition, raw milk and raw milk products are frequently contaminated with different types of *S. aureus* around the world (Tham et al., 1990; Yu et al., 2010; Rosengren et al., 2010; Jamali et al., 2015; Riva et al., 2015). Milk is a good substrate for *S. aureus* growth and enterotoxin production. Enterotoxins can retain their biological activity after pasteurization (Asao et al., 2003; Rall et al., 2008). In China, studies have reported that some *S. aureus* strains persist in powdered infant formula (Wang et al., 2012). Currently, only a few full-scale and systematic studies have been performed on *S. aureus* prevalence and contamination levels in pasteurized milk in China. This study aimed to investigate *S. aureus* contamination in pasteurized milk obtained from different Chinese cities. For this purpose, we analyzed the *S. aureus* prevalence and contamination levels, antibiotic susceptibility profiles, virulence genes, biofilm formation, biofilm-related genes, *spa* typing results, and multilocus sequence typing (MLST) results to characterize the isolates.

2. Materials and Methods

2.1 Sampling

From July 2011-June 2016, a total of 258 pasteurized milk samples were collected from supermarkets, fairs, and farmers' markets (Supplementary Table 1). The samples were obtained from 39 cities located in a total of 29 provinces and 2 directly controlled municipalities in China, which covered most of the provincial capitals of China (Supplementary Figure 1). The samples were placed in a cold box at approximately 4 ℃, tightly sealed with sterile plastic wrap, transported to an accredited laboratory, and subjected to microbiological analysis within 24 h.

2.2 Isolation and detection

The samples were qualitatively and quantitatively analyzed to detect *S. aureus*. For qualitative analysis, samples were examined according to GB 4789.30-2010 (National Food Safety Standards of China) with slight modification. The most probable number (MPN) method was used for quantitative analysis. Approximately 25 g of the food sample was homogenized in 225 mL tryptic soy broth with 10% sodium chloride (Huankai, Guangzhou, China). Subsequently, 1-mL, 0.1-mL, and 0.01-mL aliquots were transferred to tubes containing 9 mL, 10 mL, and 10 mL in triplicate with trypticase soy broth (Huankai) supplemented with 10% NaCl. The tubes were then incubated at 37 ℃ for 48 h.

A loopful of enrichment broth culture (10 μL) was streaked onto chromogenic *S. aureus* agar plates (Huankai) and incubated at 37 ℃ for 24 h. Of the colonies obtained, 1–4 pink colonies were purified on nutrient agar medium. The purified colonies were analyzed via the coagulase activity test involving freeze-dried rabbit plasma (Huankai), and the API STAPH test strips (bioMerieux, Marcy-l'Etoile, France) were then used. The MPN value was determined on the basis of the number of positive tube(s) in each of the three sets using the MPN table.

2.3 Antimicrobial susceptibility testing

The Kirby–Bauer disk diffusion method was used to test antibiotic susceptibility, and diameter interpretations were based on the protocol specified in the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2015). *S. aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were used as quality control organisms. All isolates were assessed for antimicrobial susceptibility to 24 antibiotics (Oxoid, UK): amoxycillin/clavulanic acid, ampicillin, cefepime, cefoxitin, penicillin G, ceftazidime, amikacin, gentamicin, kanamycin, streptomycin, chloramphenicol, clindamycin, erythromycin, telithromycin, ciprofloxacin, norfloxacin, tetracycline, linezolid, trimethoprim/sulfamethoxazole (1:19), rifampicin, quinupristin/dalfopristin, teicoplanin, nitrofurantoin, and fusidic acid. The CLSI zone diameter breakpoints were used to interpret the antimicrobial susceptibilities of the analyzed strains.

2.4 In vivo biofilm formation

Biofilm production was quantified using a microtiter plate assay (MPA) described previously (Vasudevan et al., 2003), with slight modifications. *S. aureus* strains were individually grown at 37 ℃ overnight in brain heart infusion broth (BHI). The overnight culture was diluted 1:100 in fresh BHI, and 200-μL aliquots of each prepared suspension were transferred into three

wells of 96-well tissue culture treated polystyrene microplates (CELLSTAR® Cell Culture Microplates, Greiner Bio-one, Frickenhausen, Germany). After cultivation at 37 ℃ for 48 h, the wells were washed three times with 200 mL sterile phosphate-buffered saline (PBS, pH 7.4) and dried at room temperature. The adherent bacterial cells were fixed with 200 μL methanol for 15 min, and the plates were emptied and left to dry overnight. The adherent cells were then stained with 1% crystal violet for 10 min and washed twice with water. The dye bound to the adherent cells was dissolved with 150 mL 95% ethanol and optical density (OD) was measured at 590 nm with a spectrophotometer (SpectroStar Nano, BMG Labtech). Uninoculated wells containing BHI served as blanks. Blank-corrected absorbance values of *S. aureus* strains were used for reporting biofilm production. Isolates were considered biofilm producers when their OD values were three times greater than the standard deviation of the mean Dc. Additionally, isolates showing biofilm-producing ability were classified as weak (Dc < OD ≤ 2*Dc), moderate (2*Dc < OD ≤ 4*Dc), or strong (OD > 4*Dc) biofilm producers.

2.5 DNA extraction and PCR

After the isolates were cultivated for at 37 ℃ 24 h in BHI, genomic DNA was extracted using a Genomic DNA Extraction kit (Magen Biotech, Guangzhou, China) according to the manufacturer's instructions. The genomic DNA concentration was determined at 260 nm by using a NanoDrop-ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, MA, USA). The *mecA/mecC* gene, which confers methicillin resistance to *S. aureus*, was detected by PCR performed using primers described previously (Perez-Roth et al., 2001; Stegger et al., 2012). PCR was also used to detect the presence of virulence genes. The detection of 18 SE genes (*sea, seb, sec, sed, see, seg, seh, sei, sej, sek, sel, sem, sen, seo, sep, seq, ser*, and *seu*), the *tsst* gene and the presence of *lukSF-PV* (*pvl*) genes was performed as previously described (Jarraud et al., 2002; Varshney et al., 2009). For adhesion genes (*clfA, clfB, fnbA, fnbB, fib,* and *cna*) and biofilm-producing genes (*icaA, icaD, icaC, bap,* and *eno*), PCR was performed according to previous studies (Zmantar et al., 2010; Artini et al., 2013; Salaberry et al., 2015; Pereyra et al., 2016). The primer sequences used in the PCR assays, expected amplified product sizes, and references are listed in Supplementary Table 2. The amplicons were stained with Goldview, loaded onto 1.5% agarose gels, electrophoresed at 120 V for 0.5 h, and visualized by a UV transilluminator gel imaging system (GE Healthcare, WI, USA). The images were saved as TIFF files for analysis.

179 **2.6 Molecular typing**

180 All the isolates were characterized by MLST and *spa* typing. The MLST analysis was based on

181 DNA sequencing of the seven housekeeping genes *arcC, aroE, glpF, gmk, pta, tpi,* and *yqil*, as

182 described previously (Enright et al., 2013). The primers spa-1113f (5′-TAA AGA CGA TCC

183 TTC GGT GAG C-3′) and spa-1514r (5′-CAG CAG TAG TGC CGT TTG CTT-3′) were used

184 for *spa* amplification (Shopsin et al., 1999). The PCR products were purified with a PCR

185 purification kit (Qiagen, Genmany) and were sequenced in both directions with Big Dye

186 fluorescent terminators on an ABI 3730XL sequencer (Applied BioSystems). For each MLST

187 locus, an allele number was assigned to each distinct sequence variant, and a distinct sequence

188 type (ST) number was attributed to each distinct combination of alleles for the seven genes.

189 STs were determined using the *S. aureus* MLST database (https://pubmlst.org/saureus/). Clonal

190 complex (CC) analysis was performed using eBURST v.3 as previously described (Feil et al.,

191 2004). The *spa* types were randomly assigned using the SpaServer website

192 (https://spaserver2.ridom.de). Sequence Type Analysis and Recombinational Tests software

193 (S.T.A.R.T. ver. 2; https://pubmlst.org/software/analysis/start2) was used to analyze the MLST

194 data.

195 **3 Results**

196 **3.1 Isolation and identification of *S. aureus* from pasteurized milk**

197 Of the 258 pasteurized milk samples, 10 samples were contaminated with coagulase-positive

198 staphylococci, and 12 isolates obtained from these positive samples were confirmed as *S.

199 aureus* by the API STAPH test. The qualitative and quantitative results for these positive

200 samples are shown in Table 1. Overall, the mean prevalence of *S. aureus* was 3.9%, and the

201 MPN values for the samples showing the highest levels (9/10) were less than 1 MPN/g.

202 Positive samples were obtained from 8 of the 39 Chinese cities included (Supplementary

203 Figure 1): 33.3% of the positive samples were from Zhanjiang and Xining cities, and 16.7%

204 were obtained from the other six cities, respectively. Besides, among the different types of

205 sampling source, the positive samples isolated from fairs was 4.2% (6/144) followed by

206 farmer`s market (3.8%, 1/26) and supermarkets (3.4%, 3/88).

207 **3.2 Antibiotic resistance**

208 Most *S. aureus* isolates showed resistance and intermediate resistance to different antibiotics

209 concentrations. The resistance patterns of *S. aureus* to the tested antimicrobial agents are

presented in Table 2. The phenotypic resistance profiles of the *S. aureus* isolates are as follows: ampicillin, 97.7%; penicillin G, 83.3%; erythromycin, 50.0%; kanamycin, 41.7%; telithromycin, 33.3%; clindamycin, 33.3%; gentamicin, 33.3%; tetracycline, 33.3%; norfloxacin, 25.0%; ciprofloxacin, 16.7%; amikacin, 16.7%; rifampicin, 16.7%; chloramphenicol, 8.3%; fusidic acid, 16.7%; ceftazidime, 8.3%; streptomycin, 8.3%; amoxycillin/clavulanic acid, 8.3%; trimethoprim/sulfamethoxazole (1:19), 8.3% and quinupristin/dalfopristin, 8.3%. All the isolates were susceptible to cefepime, cefoxitin, linezolid, and nitrofurantoin. The *mecA/mecC* gene was free in all *S. aureus* isolates. Combining with antibiotic resistance test, there was none of MRSA isolates identified. However, only one isolate was susceptible to all the antimicrobial agents tested, whereas two isolates were resistant to two antibiotics and 75.0% of isolates (9/12) were resistant to three or more classes of antibiotics which showed multi-drug resistance including one strain Sta1758 were resistant to more than 10 antibiotics.

3.3 In vitro biofilm production and presence of biofilm-related genes

The MPA results showed that all the *S. aureus* strains could produce biofilms, including four isolates (33.3%, 4/12) showing moderate and eight isolates (76.7%, 8/12) showing strong biofilm formation (Table 3). Table 3 also provides the results for the presence of *S. aureus* adhesion and biofilm-associated genes. Overall, *icaA, icaD, eno*, *clfA, clfB, fnbA, fnbB,* and *fib* were found in all 12 isolates, whereas the *bap* gene was free in all isolates. In addition, *icaC* was present in 91.7% (11/12) of the isolates and the *cna* gene in 50% (6/12). Thus, most of the biofilm-producing genes and adhesion genes were present in these *S. aureus* strains isolated from the pasteurized milk samples.

3.4 Prevalence of virulence-associated genes

The prevalence of virulence-associated genes is shown in Figure 1. Of the *S. aureus* isolates analyzed, 66.7% (8/12) carried one or more virulence-associated genes. Of the 20 virulence genes investigated, *seg, sei,* and *sem* were most frequently detected which showed 41.7% of prevalence, respectively. *sea, seb, sed, see, seu, seq, sej, ser, sek*, and *pvl* were not detected in the *S. aureus* isolates. The prevalence of the other virulence genes is as follows: *sen* (25%), *sec* (16.7%), *sel* (16.7%), *seo* (8.3%), *sep* (8.3%), *seh* (8.3%) and *tsst* (8.3%). Staphylococcal enterotoxin genes can be divided into classic SE genes (*sea, seb, sec, sed*, and *see*), *egc* cluster genes (*seg, sei, sem, sen, seo*, and *seu*), and other novel SE genes (*sep, seq, she, sej, sel, ser,* and *sek*). From the classic SE genes, only two isolates showed the *sec* gene, that is 7.7% (2/26)

242 prevalence in the detected genes. The *egc* cluster accounted for 73.1% (19/26) of the detected
243 genes, and other novel SE genes accounted for 15.4%.

244 **3.5 Molecular characterization of *S. aureus* isolates**

245 MLST analysis of the 12 isolates yielded 11 STs showing high genetic diversity. Except ST188
246 was found in two strains, the other STs were present in single strains which included ST1, ST5,
247 ST7, ST9, ST12, ST15, ST25, ST72, ST398, and ST4069. Of these, ST4069 was a newly
248 assigned ST, which found a single-locus variant (SLV) on the *aroE* gene (assigned as
249 *aroE*-472). *spa* typing showed results similar to those of MLST. Eleven *spa* types were
250 detected: t189 was found in two strains, and t177, t899, t213, t3092, t148, t091, t002, t085,
251 t078, and t034 were found in single isolates. The phylogenetic tree based on the seven
252 concatenated MLST sequences in Figure 2 shows the relatedness between these isolates.
253 ST188 correlated well with t189, which was also found in city of Xining.

254 **4 Discussion**

255 Pasteurization, or heat treatment, of milk is an important milestone in for public health and has
256 contributed to a dramatic decline in many infectious diseases. However, outbreaks associated
257 with pasteurized milk continue to occur (Ryan et al., 1987; Dalton et al., 1997; Ackers et al.,
258 2000). In China, milk has become an important component of a balanced diet, and the annual
259 per capita milk consumption increased from 9.23 kg in 1992 to 24.87 kg in 2007 (Qian et al.,
260 2011; Liu and Wang, 2013). SFP outbreaks associated with milk have occurred occasionally
261 (Dai et al., 2009). There were limited researches on the prevalence of *S. aureus* from
262 pasteurized milk in China. In the current study, 3.9% of the samples showed the presence of *S.*
263 *aureus*. Compare with other countries' studies, the prevalence of *S. aureus* from pasteurized
264 milk in our study is lower. In Brazil, Rall et al. (2008) collected 162 raw and pasteurized milk
265 samples and found 20.4% of the pasteurized milk samples were positive for *S. aureus*. In 2006,
266 Gündoğan et al. (2006) analyzed 180 raw milk, pasteurized milk, and ice cream samples; 56.6%
267 of the pasteurized milk samples were positive for *S. aureus*. However, it is maybe attribute to
268 the sample sizes, sample source or geographic locations, which showed most of these *S. aureus*
269 were isolated from farm, whereas our samples were collected from supermarkets, fairs or
270 farmer`s markets. Quantitative analysis by MPN showed that only one *S. aureus*–positive
271 sample reached a level of 10 MPN/g. In China, a limit of 100 CFU/g (mL) has been proposed
272 for *S. aureus* in milk and the highest fraction of samples with values that exceeded this limit
273 was observed at the end of food shelf-life (Helwigh and Korsgaard, 2007). Thus, the currently

274 available data, along with our study, indicate that levels of *S. aureus* in pasteurized milk in
275 China are not very high.

276

277 Staphylococcal enterotoxins are responsible for most SFP outbreaks (Dinges et al., 2000, Loir
278 et al., 2003). This could account for retention of biological activity after pasteurization. This
279 study was investigated the 18 SE genes among pasteurized milk related *S. aureus* isolates and
280 found that 66.7% of the isolates carried one or more SE genes, suggesting the risk of *S. aureus*
281 contamination in pasteurized milk in China. This rate is higher than that reported in a previous
282 study (Chao et al., 2015) in which the prevalence of 18 SE genes was studied in *S. aureus*
283 isolates obtained from different origins in China, showing that 54.4% of those isolates harbored
284 SE genes. Generally, the *sea* gene has been the most common gene in the SFP outbreak isolates
285 tested, followed by the *sed* gene from classic SE genes (Kérouanton et al., 2007), but they were
286 not detected in the current study. *sec* was the only classical SE gene detected in our study,
287 which is the most commonly occurring SE in milk (Lindqvist et al., 2002; Scherrer et al., 2004;
288 Aragon-Alegro et al., 2007). In 2002, an outbreak of community-acquired foodborne illness
289 caused by MRSA was attributed to SEC-containing coleslaw, which was produced in the
290 United States (Jones et al., 2002). In addition, we observed higher prevalence of *seg-sei-sem*
291 among the isolates in our study, these SE genes belonged to the *egc* cluster, which is widely
292 distributed in clinical isolates and acts as a putative nursery of enterotoxin genes (Jarraud et al.,
293 2001; Wu et al., 2018b). Thus, the hazard posed by these isolates harboring SE genes should
294 not be ignored.

295

296 In the current study, all *S. aureus* strains obtained from pasteurized milk samples could produce
297 biofilms and showed moderate or strong biofilm production capability. In addition, various
298 genes have been reported to be involve in biofilm formation, of these genes the *ica* group genes
299 especially *icaA* and *icaD* (Arciola et al., 2001) and the other group such as *fnbA, fnbB, cna* and
300 *eno* genes that encode MSCRAMMs proteins (Atshan et al., 2012) are noticeable. Most of
301 these genes were present in the *S. aureus* strains isolated from our samples. This result were
302 similar with the research by Atshan et al. (2012) who detected 9 MSCRAMM and 4 biofilm
303 related genes in the MSSA and MRSA isolates from clinically patients by PCR method and
304 found *fnbA, eno, ebpS, clfA, clfB, icaA, icaD, icaB*, and *icaC* genes were detected in 100% of
305 isolates. Furthermore, *fib* and *cna* were detected in 90% and 46.6% MSSA isolates,
306 respectively, which is in agreement with our result. In our study, the *bap* gene was free in all *S.*
307 *aureus* isolates. Many studies have reported that the *bap* gene was not detected or very low
308 rates in the solutes studied (Melchior et al., 2007; Szweda et al., 2012; Khoramrooz et al., 2016;

Pereyra et al., 2016; Felipe et al., 2017). Although there was inconclusive evidence for the correlation between phenotypic tests and the detection of specific genes associated with adhesion and biofilm formation, the presence of *icaA, icaD, icaC, fnbA, clfA, clfB, fnbA, fnb,* and *fib* genes indicated a significant association with biofilm formation in this study. The biofilm production in isolates with negative results for any of the genes studied may be attributable to other biofilm-related genes.

Antimicrobial resistance was noted in pasteurized milk related *S. aureus* isolates. Most of the isolates were resistant to at least one antimicrobial agent. However, resistance to ampicillin and penicillin was higher than that reported in many previous studies involving *S. aureus* isolates from dairy samples (Rosengren et al., 2010; Jamali et al., 2015; Riva et al., 2015). We also observed that 25.0% of the isolates were resistant to multiple antibiotics such as ampicillin, gentamicin, kanamycin, clindamycin, erythromycin, and telithromycin. These were similar to the study by Jamali et al.' investigationt, which showed the resistance profiles of *S. aureus* isolates from raw milk and dairy products for tetracycline, penicillin, clindamycin, erythromycin, streptomycin, kanamycin, chloramphenicol, and gentamicin (Jamali et al., 2015). Furthermore, many isolates were found intermediate-resistant to the selected antibiotics in this study. The reduced antibiotic susceptibility of these antibiotics suggests that these bacteria may be exposed to such antimicrobials for a long time (Howden BP et al., 2010). Thus, it should draw public attention and controlled use of antimicrobials would limit the emergence of drug-resistant bacteria.

MLST and *spa* genotyping have shown a highly clonal population structure for *S. aureus* in many studies (Kanika et al., 2011; Fetsch et al., 2014; Basanisi et al., 2017). The *S. aureus* MLST database (https://pubmlst.org/bigsdb?db=pubmlst_saureus_isolates&page=profiles) currently has 5024 different STs. However, some STs are specific to a particular source. For example, ST398 occurs in livestock-associated *S. aureus*; ST5, ST45, and ST239 have been detected in hospital-associated *S. aureus* infections; and ST1, ST8, ST30, and ST59 are often related to community-associated *S. aureus* infections (Bens et al., 2006; Cui and Li, 2009; Neela et al., 2009; Skov, 2009; Deleo et al., 2010). Generally, *spa* typing showed highly concordant with MLST. In the current study, MLST and *spa* typing enabled classification of pasteurized milk related *S. aureus* isolates into 11 types (ST188-t189, ST1-t177, ST9-899, ST12-t213, ST72-t3092, ST7-t091, ST5-t002, ST15-t085, ST25-t078, ST398-t034, and ST4069-t148). Most of these STs were detected in various of samples and relevant to a variety of clinical *S. aureus* infections (Krziwanek et al., 2009; Lee et al., 2011; Yu et al., 2012;

Schaumburg et al., 2015). Therefore, the hazards of these strains for consumers cannot be ignored.

To conclude, our results provide information regarding the genetic background of *S. aureus* strains obtained from pasteurized milk samples in China. To our knowledge, our study is the first systematic investigation of prevalence and contamination levels for *S. aureus* isolated from pasteurized milk samples in China. Different source strains showed different types in this study. These isolates had virulence potential, which half of them carried enterotoxin genes and infection-related molecular type. The resistance patterns can help determine more appropriate treatments for human infections in China. All the isolates had moderate or strong biofilm-production capability, and most of them harbored biofilm-related genes, indicating that these potentially virulent bacteria could persist in the food-manufacturing environment.

Conflict of Interest Statement

Funding

Reference

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582 **Table**

583 Table 1 Prevalence and contamination levels of *Staphylococcus aureus* in pasteurized milk
584 samples from China

Positive number	Positive sample	Sampling site	Source	Quantitative methods MPN values (MPN/g)	Qualitative methods
1	ZJJ376	Zhanjiang	Fair	0.92	+
2	ZJC1758	Zhanjiang	Supermarket	<0.3	+
3	FZJ2233	Fuzhou	Fair	24	+
4	XMJ2333	Xiamen	Fair	0.36	+
5	LZN1142	Lanzhou	Farmer`s market	<0.3	+
6	XNC3008	Xining	Supermarket	0.3	+
7	XNJ3032	Xining	Fair	0.3	+
8	XGJ3532	Hongkong	Fair	0.75	+
9	GYC3608	Guiyang	Supermarket	<0.3	+
10	HHHTJ4032	Huhehaote	Fair	0.74	+

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587 Table 2 Antimicrobial susceptibility tests for *Staphylococcus aureus* isolates obtained from
588 pasteurized milk samples from China.

	Antibiotics	*S. aureus* (n = 12)		
		No. of resistant strains (%)	No. of intermediate-resistance strains (%)	No. of susceptible strains (%)
β-Lactams	Amoxycillin/clavulanic acid	1 (8.3%)	-	11 (97.7%)
	Ampicillin	11 (97.7%)	-	1 (8.3%)
	Cefepime	0 (0%)	0 (0%)	12 (100%)
	Cefoxitin	0 (0%)	-	12 (100%)
	Penicillin G	10 (83.3)	-	2 (16.7%)
	Ceftazidime	1 (8.3%)	2 (16.7%)	9 (75.5%)
Aminoglycosides	Amikacin	2 (16.7%)	2 (16.7%)	8 (66.7%)
	Gentamicin	4 (33.3%)	-	8 (66.7%)
	Kanamycin	5 (41.7%)	2 (16.7%)	5 (41.7%)
	Streptomycin	1 (8.3%)	8 (66.7%)	3 (25.0%)
Phenicols	Chloramphenicol	1 (8.3%)	3 (25.0%)	8 (66.7%)
Lincosamides	Clindamycin	4 (33.3%)	1 (8.3%)	7 (58.3%)
Macrolides	Erythromycin	6 (50.0%)	1 (8.3%)	5 (41.7%)
	Telithromycin	4 (33.3%)	3 (25.0%)	5 (41.7%)
Fluoroquinolones	Ciprofloxacin	2 (16.7%)	5 (41.7%)	5 (41.7%)
	Norfloxacin	3 (25.0%)	3 (25.0%)	6 (50.0%)
Tetracyclines	Tetracycline	4 (33.3%)	-	8 (66.7%)
Oxazolidinones	Linezolid	0 (0%)	0 (0%)	12 (100%)
Ansamycins	Rifampicin	2 (16.7%)	1 (8.3%)	9 (75.0%)
Sulfonamides	Trimethoprim/sulfamethoxazole (1:19)	1 (8.3%)	0 (0%)	11 (97.7%）
Quinolones	Quinupristin/dalfopristin	1 (8.3%)	0 (0%)	11 (97.7%）
Glycopeptides	Teicoplanin	0 (0%)	2 (16.7%)	10 (83.3%)
Nitrofurantoins	Nitrofurantoin	0 (0%)	0 (0%)	12 (100%)
	Fusidic acid	2 (16.7%)	-	10 (83.3%)

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Table 3 Biofilm-formation ability and biofilm-associated genes in *Staphylococcus aureus* isolates obtained from pasteurized milk samples

No.	*S. aureus* isolates	Biofilm production assay	Biofilm production ability[a]	biofilm-producing genes					adhesion genes					
				icaA	*icaD*	*icaC*	*bap*	*eno*	*clfA*	*clfB*	*fnbA*	*fnbB*	*fib*	*cna*
1	Sta376	1.4715	+++	+	+	+	-	+	+	+	+	+	+	+
2	Sta1758	0.5147	++	+	+	+	-	+	+	+	+	+	+	-
3	Sta2233-0	1.2700	+++	+	+	+	-	+	+	+	+	+	+	-
4	Sta2333-0	0.8515	++	+	+	+	-	+	+	+	+	+	+	-
5	Sta1142	1.1548	+++	+	+	+	-	+	+	+	+	+	+	+
6	Sta3008C2	0.5417	++	+	+	+	-	+	+	+	+	+	+	+
7	Sta3032	0.6850	++	+	+	+	-	+	+	+	+	+	+	+
8	Sta3032C3	1.1167	+++	+	+	-	-	+	+	+	+	+	+	-
9	Sta3532	1.0995	+++	+	+	+	-	+	+	+	+	+	+	-
10	Sta3608	2.5659	+++	+	+	+	-	+	+	+	+	+	+	-
11	Sta4032B2	2.6347	+++	+	+	+	-	+	+	+	+	+	+	+
12	Sta4032B3	2.7753	+++	+	+	+	-	+	+	+	+	+	+	+

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592 a: Quantification of biofilm formation by optical density (OD) analysis: (+++): strong biofilm producers ($OD_{590} > 0.88$), (++): moderate biofilm producers (0.44 >
593 $OD_{590} > 0.88$), (+): weak biofilm producers (0.22 > $OD_{590} > 0.44$).
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596 Fig. 1 Staphylococcal toxin gene profiles of *Staphylococcus aureus* isolates obtained from

597 pasteurized milk

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600 Fig. 2 Unweighted pair group method with arithmetic mean (UPGMA) tree of the 7 multilocus

601 sequence typing loci of *S. aureus* isolates obtained from pasteurized milk. This tree was

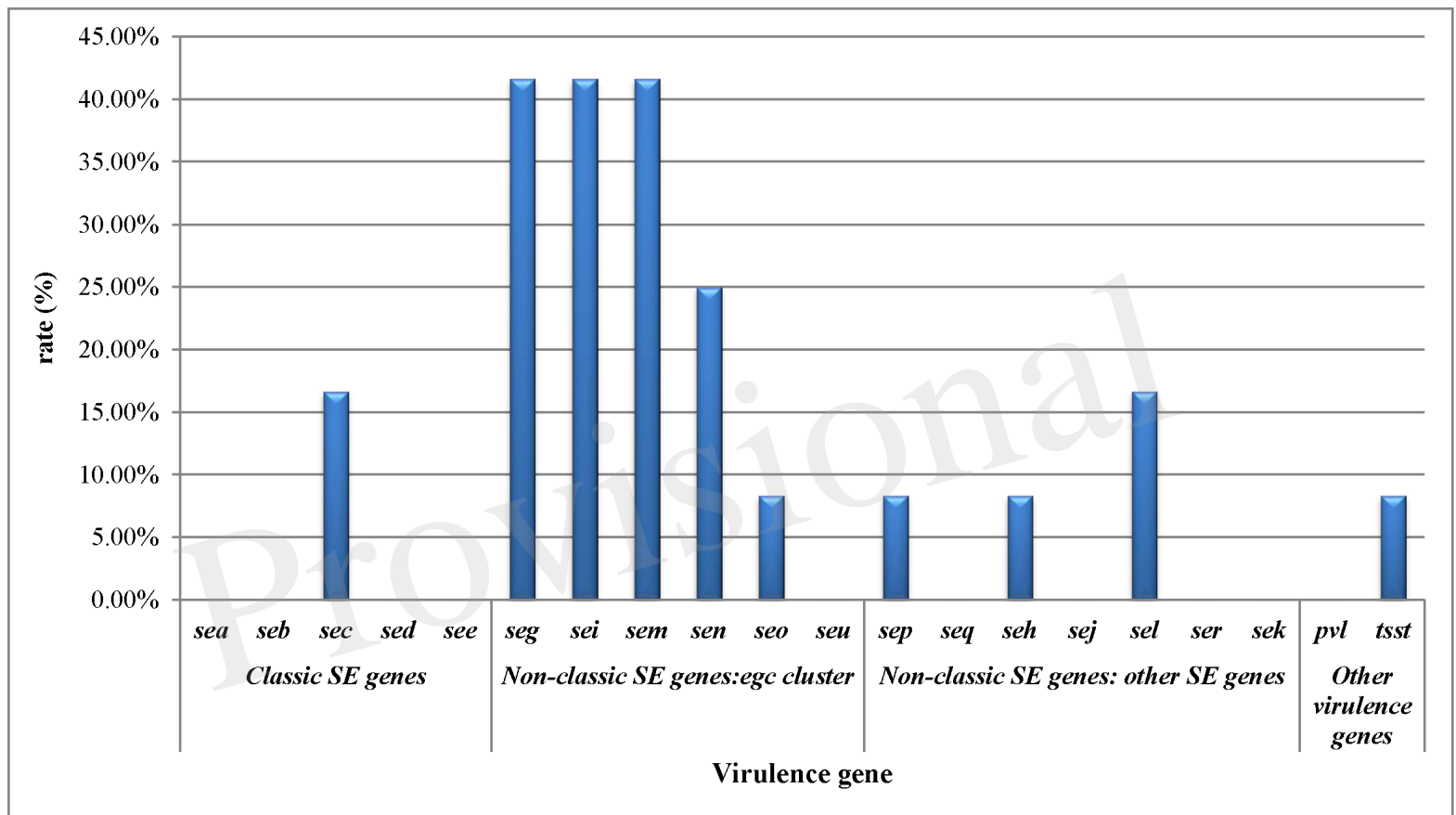602 generated using S.T.A.R.T. (version 2).

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Figure 01.TIF

Figure 02.TIF

Strains	ST	*spa*-type	Sample origin	Antibiotic profiles	SEs gene
Sta3008C2	188	t189	Xining	AMP-P	-
Sta3032	188	t189	Xining	AMC-AMP-P	-
Sta4032B2	1	t177	Huhehaote	AMP-CN-K-DA-E-TEL-TE-RD-SXT	*seh*
Sta1758	9	t899	Zhanjiang	AMP-P-S-C-DA-E-TEL-CIP-NOR-TE-QD	*seg-sei-sem-sen*
Sta4032B3	12	t213	Huhehaote	AMP-P-CN-K-DA-E-TEL-TE	*sec-sel*
Sta3532	72	t3092	Xianggang	AMP-P-AK-CN-K-RD-FD	*seg-sei-sem-seu*
Sta376	4069	t148	Zhanjiang	-	*sec-seg-sei-sem-sen-tsst*
Sta3608	7	t091	Guizhou	AMP-P-CAZ-AK-K-NOR-TE-FD	*sep*
Sta2233-0	5	t002	Fuzhou	AMP-P-CN-K-DA-E-TEL-CIP-NOR	*seg-sei-sem*
Sta2333-0	15	t085	Xiamen	AMP-P-E	-
Sta3032C3	25	t078	Xining	AMP-P-E	*seg-sei-sem-sen*
Sta1142	398	t034	Lanzhou	AMP-P	-

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