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Prevalence and characterization of *Staphylococcus aureus* and *Staphylococcus argenteus* in chicken from retail markets in China

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ABSTRACT

Staphylococcus aureus (*S. aureus*) is one of the most common pathogens causing both human and animal infections. Transmission of *S. aureus* to humans via contaminated food continues to be a health public concern. In the present study, 104 strains including eight MRSA strains were identified from 507 chicken samples (20.5%) in three cities of China. These strains harbored the highest resistance against penicillin (91.4%), followed by tetracycline (64.4%), erythromycin (53.5%), and kanamycin (32.7%). We used *spa* typing to classify these isolates into 28 types belonging to two lineages including the predominant type t112 (n = 26) and four newly identified *spa* types. Among the 104 strains, six carried the CRISPR-Cas system, a prokaryotic immune system which protects against foreign genetic elements. Interestingly, *rpoB* gene sequencing demonstrated that these six initially designated ST2250 strains were in fact *S. argenteus*, a novel *Staphylococcus* species genetically closely related to *S. aureus*. Three *Staphylococcus* CRIPSR types containing ten spacers identified in these strains have been reported in CRISPR-positive *S. aureus*. Additionally, 80% of the spacers showed homology to *S. aureus* phages demonstrating that these conserved spacers were closely related to the phages in the environment of *S. argenteus*. We speculated that the identical CRISPR types and spacers in both *S. argenteus* and *S. aureus* have resulted via exchange of mobile elements between these two species. Emergence of food-borne ST2250 *S. argenteus* is a potential threat to human public health.

1. Introduction

Staphylococcus aureus (*S. aureus*) is an important zoonotic pathogen, which can infect both humans and animals. It is widely distributed in nature and is present in air, water and feed; it also exists on the surface of the human body, in the nasal cavity, on animal fur, and in the digestive tract among other sites. *S. aureus* has been responsible for several infectious diseases including tissue and skin infections, pneumonia, sepsis, mastitis, arthritis, and soft tissue infections (David & Daum, 2010; Tong, Davis, Eichenberger, Holland, & Fowler, 2015a). Livestock products can act as a source of *S. aureus* zoonotic infections, and handling or consuming of contaminated food could potentially result in transmission to humans (Feingold et al., 2012; Papadopoulos et al., 2018; Peton & Le Loir, 2014).

Recent studies have shown that a novel staphylococcal species named *S. argenteus* has diverged from *S. aureus*, and has been reported in New Zealand, Asia, Europe and South America (Aung et al., 2017). MSHR1132 was the type strain of *S. argenteus* isolated from a patient blood in Australia, and then, many human clinical isolates of ST2250 (multi-locus sequencing type, MLST) were detected globally (Moradigaravand et al., 2017). The ST2250 *S. argenteus* isolates have been found to be distributed in the South Asia (Myanmar and Thailand) and Europe (France and Belgium), particularly in Thailand, where 83% (57/68) of human clinical isolates from patients with community-associated invasive infections at multiple hospitals in Thailand were ST2250 isolates carried the CRISPR-Cas system (Moradigaravand et al., 2017). Three *S. argenteus*-related food poisoning outbreaks and one 12-year-old boy of purulent lymphadenitis caused by ST2250 *S. argenteus* have also been reported in Japan recently (Ohnishi et al., 2018; Suzuki et al., 2017; Wakabayashi et al., 2018). *S. argenteus* shares many

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characteristics of genome sequence with *S. aureus*, including virulence genes, pathogenicity islands, bacteriophages, antimicrobial resistance genes (Chantratita et al., 2016; Hansen et al., 2017; Tong et al., 2015b; Tunsjø, Kalyanasundaram, Charnock, Leegaard, & Moen, 2018), which may be closely related to its infection in patients. Genomic comparison of geographic distributed ST2250 *S. argenteus* also revealed that the pathogen probably had undergone a recent host adaption to cause human infections (Zhang et al., 2017). In addition, staphylococcal enterotoxin genes (*seb*, *sec*) were also detected in clinical *S. argenteus*, which reflected that the pathogen has the potential to cause food poisoning and other severe infections (Ohnishi et al., 2018; Suzuki et al., 2017).

Due to the concern of the Chinese government over food safety and control, substantial data regarding prevalence and characteristics of *S. aureus* including MRSA in retail foods in China is needed (Wang et al., 2013, 2014). In 2010, the overall prevalence of *S. aureus* in whole raw chickens around China was 24.2%, and 1.7% samples were positive for MRSA (Wang et al., 2013). However, few reports regarding this have been published during recent years. The aim of the present study was to determine the prevalence, antibiotic resistance, and molecular characteristics of *S. aureus* and MRSA in retail chicken sold at large supermarkets and farmers' markets in three Chinese cities during 2016. In addition, we report, for the first time, the prevalence and characteristics of the CRISPR-Cas system in the food-borne ST2250 *S. argenteus* isolates.

2. Materials and methods

2.1. Chicken samples collection

To evaluate the prevalence of *S.* aureus in chicken at retail markets in some areas of China, a total of 507 raw chicken carcass samples was randomly collected from seven supermarkets, 10 farmers' markets, two wholesale markets, one slaughter house, and one vegetable/food market between July 2016 and November 2016. Among 507 samples, 274 including 158 raw chickens and 116 chilled chickens were collected from Chongqing city in southwest China; 195 and 38 chilled chicken samples were collected from the southeastern cities of Lianshui and Yangzhou, respectively. All of the samples were aseptically placed into sterile Whirl-Pak bags (Nasco, USA), labelled, stored on ice and immediately transported to the laboratory in Yangzhou University for microbiological analysis within 24 h.

2.2. Bacterial isolation and identification

Isolation and Identification of *S. aureus* was performed by the following experimental procedure with some modifications (Tang et al., 2017). The meat samples were manually rinsed in 500 mL buffered peptone water per kilogram and agitated for 5 min, and then 10 mL of the sample broth was enriched in 90 mL trypticase soy broth (TSB, OXOID, United Kingdom) containing 6.5% NaCl and incubated at 37 °C for 24 h. Next, a loopful (approximately 10 μL) of the culture was inoculated onto Baird Parker agar (BPA, OXOID) and incubated at 37 °C for 20–24 h. One to four of the suspected colonies on the BPA plate were subjected to the tube coagulase test in freeze dried rabbit plasma with EDTA (Sigma, USA). Isolates that passed the coagulase test were considered for further identification via amplification of the species-specific *nucA* gene using a previously described primer set (Louie, Matsumura, Choi, Louie, & Simor, 2000). Each *S. aureus* strain was preserved at – 80 °C in TSB with 20% glycerol. The *rpoB* sequencing was performed to differentiate *S. argenteus* from *S. aureus* using primers and conditions as previously described (McDonald et al., 2005). Briefly, the *rpoB* sequences were analyzed with the Bionumerics 7.0 software (Applied Mathematics, Belgium) to construct a similarity dendrogram using the multiple sequence alignment and the unweighted pair group method with arithmetic averages (UPGMA). The published *rpoB* sequences from *S. aureus* and *S. argenteus* (GenBank: NC_016941.1; GCA_000752055.1; NC_018608.1; KB822075.1) were used as controls. The *S. argenteus* isolates were further identified by inoculation on salt egg yolk agar plate to produce white color clone because the bacteria lack the genes for production of staphyloxanthin (Holt et al., 2011).

2.3. Antimicrobial susceptibility testing

All antimicrobial susceptibility tests were performed using the disk-diffusion method following the standards enacted by the Clinical and Laboratory Standards Institute (CLSI) in 2016. Thirteen antimicrobial agents (OXOID, England) used in this study were (dose in μg/disk): penicillin (P, 10 units), cefoxitin (FOX, 30), teicoplanin (TEC, 30), gentamicin (CN, 10), kanamycin (K, 30), erythromycin (E, 15), tetracycline (TE, 30), ciprofloxacin (CIP, 5), nitrofurantoin (F, 300), clindamycin (DA, 2), chloramphenicol (C, 30), rifampin (RD, 5) and trimethoprim (W, 5). *S. aureus* ATCC25923 was used as the quality control strain.

2.4. spa typing and CRISPR analysis

Genomic DNA of all the isolates was extracted using the DNeasy blood and tissue kit (Qiagen, Germany). Sequence typing of the *S. aureus* protein A (*spa* typing) was then performed in these isolates as previously described (Lozano et al., 2011). Briefly, PCR products of the *spa* locus were sequenced using an ABI 3130 Genetic Analyzer (ABI, Calif) by Nanjing Genscript Corporation (Nanjing, China). The sequences were then submitted to the Ridom Spa Server (http://spa.ridom.de/) to determine the *spa* types based on the number and arrangement of tandem repeat sequences. Newly detected *spa* types were referred based on the arrangement of repeated sequences until a new name was suggested by the database curators. The genetically relationship of all the isolates was finally displayed in the form of a phylogenic tree based on *spa* types using the software MEGA 7.0.

PCR analysis was used to detect the CRISPR-Cas system in all of the *S. aureus* isolates as previously described (Li et al., 2016). The primers used to detect *cas6*, CRISPR1, and CRISPR2 were designed according to the published genome sequences of CRISPR-positive *S. aureus* at GenBank, NCBI (Supplementary Table S1). The sequences of CRISPR1 and CRISPR2 loci were subjected to the CRISPRtionary (http://crispr.upsud.fr/CRISPRcompar/Dict/Dict.php) analysis to identify the arrangement of spacers. The spacers were then blasted with the spacers previously published (Li et al., 2016), and newly detected spacers were named as new ones. In addition, homology of the spacers to plasmid or phage sequences was analyzed using BLASTn.

2.5. SCCmec typing, MLST, and PVL detection of MRSA and CRISPR-positive strains

All of the isolates including cefoxitin-resistant strains were subjected to multiplex PCR of *mecA* and *mecC* (Louie et al., 2000) for confirmation of the MRSA phenotype (Supplementary Table S1). Confirmed MRSA stains were further characterized using MLST, SCC*mec* typing, and PVL (Panton-Valentine Leukocidin, PVL) detection. The MLST was performed based on sequencing of seven housekeeping genes (*arc*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL*) to evaluate evolutionary relationships of the MRSA and/or CRISPR-positive isolates as previously described (Pu, Han, & Ge, 2009). Alleles at the seven loci were sequenced and assigned by comparing the sequences at each locus with those of the known alleles in the *S. aureus* MLST database (http://saureus.mlst.net/). PVL detection and SCC*mec* typing of these isolates was performed using a multiplex PCR method based on the PVL gene and the *mec* complex (Zhang, McClure, Elsayed, Louie, & Conly, 2005). The Clonal Complex (CC) of the MRSA strains was classified following previously described methods. (Suzuki, Matsumoto, Takahashi, Hayakawa, & Minagawa, 2009). The MLST and SCC*mec* typing were