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Genetic diversity and virulence potential of *Staphylococcus aureus* isolates from raw and processed food commodities in Shanghai

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ABSTRACT

The risk of zoonotic transmission to humans highlights the need to understand the molecular ecology of *Staphylococcus aureus* in foods. In this study, 142 *S. aureus* isolates obtained from various raw and processed foods from Shanghai, China were characterized to determine their genetic diversity and virulence gene content. A total of 16 clonal complexes (CCs), 34 staphylococcal protein A (*spa*) types, and 6 accessory gene regulator (*agr*) allelic groups were identified and analyzed among the 142 *S. aureus* isolates. Among these, the genotype CC188-t189-*agr* I was the most prevalent, constituting 28.2% of all isolates. The presence of virulence genes encoding 20 staphylococcal enterotoxins (*se*), toxic shock syndrome toxin (*tsst1*), exfoliative toxins (*eta*, *etb*, and *etd*), Panton–Valentine leukocidin (*lukS*-PV and *lukF*-PV), as well as methicillin resistance gene (*mecA*), was determined by PCR. Of these *S. aureus* isolates, 72.5% harbored toxin genes, in which the most frequent toxin gene was *sep* (43.7%), followed by *sej* (26.1%) and *pvl* (21.1%). In contrast, *see*, *ses*, *set*, *tsst1*, *etb*, and *etd* were not found in any of the isolates tested. Eight *S. aureus* isolates (5.6%, 8/142), seven from raw milk and one from frozen food, were *mecA* positive and resistant to oxacillin, thus were MRSA. The 142 *S. aureus* isolates displayed 52 different toxin gene profiles. Although no direct association was found between toxin gene profile and the *S. aureus* genotype, the isolates belonging to CC5, CC9, CC20, CC50, and CC72 clonal lineages in general carried more toxin genes (>5) compared with the isolates in other CCs. It was also revealed that raw milk and raw meat were the major sources of isolates containing multiple toxin genes. *S. aureus* isolates from food that were genetically highly related, displayed diverse toxin gene profiles, implying the significant role of horizontal gene transfer in the emergence of highly toxigenic *S. aureus* isolates.

1. Introduction

Staphylococcus aureus is the leading cause of nosocomial infections and often associated with food poisoning worldwide (Argudin et al., 2010). The recent emergences of hospital-acquired methicillin resistant *S. aureus* (HA-MRSA), community-acquired MRSA (CA-MRSA), as well as livestock-associated MRSA (LA-MRSA) pose a serious threat to public health. *S. aureus* is generally considered a host-specific organism; however, recent studies documented that LA-MRSA were able to colonize and cause invasive disease in humans (Cui et al., 2009; Graveland et al., 2011; Koeck et al., 2013; Neela et al., 2009). This risk of zoonotic transmission to humans demands our deep understanding of the ecology of *S. aureus* in food and food-related environments (Walther et al., 2009).

S. aureus produces a wide variety of protein toxins such as enterotoxins (SEs), toxic shock syndrome toxin1 (TSST-1), exfoliative toxins, and Panton–Valentine leukocidin (PVL). TSST-1 diminishes the immune response of a colonized host and is responsible for toxic shock syndrome (Bergdoll et al., 1981). SEs are the major cause of staphylococcal food poisoning, which has symptoms including nausea, violent vomiting, and abdominal cramping, with or without diarrhea. To date, in addition to the five classical enterotoxin types (SEA-SEE), 16 new types SEs of SEIs (SEG-SEIV) have been described (Argudin et al., 2010; Omoe et al., 2013). Many genes encoding enterotoxins are known to be associated with pathogenicity islands where they are grouped either as a gene cluster or organized as an operon. In addition, these genes are often located on mobile genetic elements (MGEs), including prophages, plasmids or transposons (Novick et al., 2010; Omoe et al., 2003; Ono et al., 2008). Similarly, the genes encoding PVL, associated with severe pneumonia and soft tissue infections, are also located on a prophage (Boyle-Vavra and Daum, 2007). Furthermore, the genes (*eta*, *etb*, and *etd*) encoding exfoliative toxins, responsible for staphylococcal scalded skin syndrome, are also located on MGEs (Novick, 2003). Horizontal transfer of MGEs among *S. aureus* strains not only promotes the

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emergence of "super" bugs, but also accelerates the dissemination of pathogenic *S. aureus* strains among animals as well as in humans (Uhlemann et al., 2012).

Raw and processed meat, raw milk, and dairy products have been reported to be the major food sources associated with *S. aureus* food poisoning (Kerouanton et al., 2007; Waters et al., 2011). Extensive studies have been carried out to characterize the genotypes and virulence properties of *S. aureus* strains isolated from milk and meat (Aydin et al., 2011; Casagrande Proietti et al., 2010; Hata et al., 2010; Pereira et al., 2009). It has been reported that over a half of *S. aureus* isolates from meat or milk are enterotoxigenic. Furthermore, it was suggested that certain *S. aureus* lineages were specifically associated with milk, such as CC97 (Hata et al., 2010). *S. aureus* is widespread in natural environments including both food and food-processing environments. In this study, we evaluated the genetic diversity and the virulence potential of *S. aureus* isolates obtained from raw milk, fresh meat, processed soybean product, frozen foods, and fresh vegetables/fruits in Shanghai, China. We then examined the possible link of virulence gene content with *S. aureus* genotypes and food categories.

2. Material and methods

2.1. Isolation and identification of S. aureus

A total of 607 food samples were randomly collected for *S. aureus* isolation during a two year period (July 2010 to October 2012) in Shanghai, China. Briefly, 248 raw milk samples were obtained from bulk tanks at 12 dairy farms with at least three samples being taken from the each bulk tank. Fresh meat, frozen foods, processed soybean products, and fresh vegetables and fruits were randomly purchased from 14 local grocery stores in three districts (Minhang, Xuhui, and Baoshan) in Shanghai. Fresh meat samples included 31 fresh beef samples, 68 pork samples, and 29 chicken samples. The 61 frozen food samples included various quick-frozen dumplings with meat and vegetable fillings which were stored under the freezing conditions for transportation and sale. Processed soybean product samples included fresh tofu (35), tofu skin (16), and dried tofu (17). The 102 fresh vegetable and fruit samples were various leafy greens, mushrooms, apples, and pears. Isolation and identification of *S. aureus* were performed according to China's National Technical Standard GB4789.10-2010. After incubation at 37 °C for 24 h on Baird–Parker agar plates with 5% egg yolk and tellurite (BPA, Beijing Land Bridge Technology Ltd., Beijing, China), up to two presumptive colonies (black colonies surrounded by 2–5 mm clear zones) were selected from *S. aureus*-positive food sample (Wang et al., 2012). Putative *S. aureus* isolates were further tested for hemolytic and coagulase activities, followed by PCR to identify the *S. aureus* specific gene *nuc1* (Brakstad et al., 1992). All *S. aureus* isolates were routinely grown at 37 °C in tryptic soy broth (Becton Dickinson, Sparks, MD).

2.2. Detection of staphylococcal toxin genes

Primers targeting 25 toxin genes, including 20 enterotoxin genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq*, *ser*, *ses*, *set and seu*), the toxic shock syndrome toxin (*tsst1*), three exfoliative toxin genes (*eta*, *etb*, and *etd*), and the Panton–Valentine leukocidin encoding genes (*lukS-PV* and *lukF-PV*) are listed in Table S1 (Supporting information). PCR primers were synthesized at Sangon Co., Ltd (Shanghai, China). Genomic DNA of each *S. aureus* isolate was purified using a modified cetyltrimethylammonium bromide method as described previously (Xie et al., 2011). PCR was performed in a 25 μL volume containing 50 ng of genomic DNA, 1U of Taq DNA polymerase (Fermentas Inc., Glen Burnie, MD, USA), 1.5 mM $MgCl_2$, 0.4 μM of each primer, and 0.2 mM of each dNTP, using an Eppendorf PCR system (Eppendorf, Germany). Strains with known toxin genes were used as positive controls: strain ATCC8095 for genes *sea*, *sed*, *sej*, *sek*, *seq*, and *ser*; strain ATCC14458 for the *seb* gene; strain ATCC27664 for the *see* gene; strain ATCC27661 for genes *seg*, *sei*, *sem*, *sen*, and *seo*; strain A176 for genes *seh* and *agr* I; strain C299 for genes *sep*, *mecA*, and *agr* II; strain O114 for genes *tsst1*, *sec*, *sel*, *seu*, and *agr* III; strain F104 for genes *eta* and *agr* IV; and strain O143 for *etd* gene (Xie et al., 2011).

2.3. Detection of methicillin-resistant S. aureus (MRSA) isolates

All isolates were tested for methicillin resistance by the disk diffusion method according to the Clinical Laboratories Standards Institutes guidelines (CLSI 2012). Oxacillin disks (1 μg) were used for detecting methicillin-resistant isolates. *S. aureus* ATCC 25923 was used as a control. The *mecA* gene, which has been shown to confer methicillin resistance to *S. aureus* (MRSA), was also detected by PCR using primers described previously (Perez-Roth et al., 2001).

2.4. Multilocus sequence typing (MLST)

MLST was carried out as described previously (Enright et al., 2000). Briefly, seven *S. aureus* housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL*) were amplified by PCR and bi-directional DNA sequencing was performed for all of the PCR products. DNA sequences were assembled in SeqMan (Lasergene 8, DNASTAR, Madison, WI, USA). The allelic profiles (represented by ST) were determined using the sequences of all seven housekeeping genes described above using the default parameters listed on the MLST home page (http://www.mlst.net/). The clonal complex (CC) analysis was performed in eBURST v.3 as previously described (Feil et al., 2004). CCs were composed of STs that shared at least six of the seven housekeeping genes with a predicted ancestral ST differing from the largest number of other STs at only one single locus.

2.5. spa typing

The *spa* typing was performed as previously described (Harmsen et al., 2003). Briefly, the repeat-containing region of the staphylococcal protein A gene (*spa*) was amplified by PCR followed by DNA sequencing of the PCR products. The *spa* repeats and types were determined using the Ridom Spa Server (http://spaserver.ridom.de/). If a *spa* repeat did not match any *spa* types in the database, the sequence of the *spa* was identified as a new *spa* type.

2.6. agr genotyping

The *agr* allele types (I–IV) were determined by multiplex PCR as described by Gilot et al. (2002). These primers yield a PCR product of 441 bp, 575 bp, 323 bp, or 659 bp corresponding to *agr* group I, II, III, and IV, respectively. For those isolates that yielded no amplification products by *agr* multiplex PCR, a *Sca*I RFLP analysis was performed as described previously (Cafiso et al., 2007). Briefly, the 2583 bp *agr* locus was amplified by PCR, followed by digestion with *Sca*I (Fermentas Life Science, China). The resulting DNA fragments were separated using gel electrophoresis on 1.0% agarose gels, stained with ethidium bromide and visualized under UV light. If the *agr* primers yielded a PCR product of a different size (i.e., not 2583 bp), DNA sequencing was performed to determine if the PCR product was a result of non-specific amplification.

2.7. Statistical analysis

Statistical analysis was computed using SPSS v.18.0 (SPSS Inc., Chicago, IL, USA). Pearson's chi-square test (two-tailed) was used to analyze the difference of the distribution of toxin genes among isolates from various food isolates. The difference is considered significant if the p value is less than 0.05.