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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.

3. Results

3.1. Isolation and identification of S. aureus

Of the 607 food samples, 117 (19.3%) were positive for *S. aureus*, including 50 (20.2%) of the 248 raw milk samples, 36 (28.1%) of the 128 fresh meat samples, 13 (21.3%) of the 61 frozen food samples, 6 (8.8%) of the 68 processed soybean products, and 12 (11.8%) of the 102 vegetables and fruits (Table 1). In order to obtain more genetic diversity of *S. aureus*, up to two isolates per sample were selected for further analysis. But if isolates from a single food sample had the same genotype determined by MLST, *spa* type, *agr* type, as well as an identical toxin gene profile, they were considered a single strain. Based on these criteria, a total of 142 *S. aureus* isolates were obtained from the 117 *S. aureus*-positive food samples, including 58 from raw milk, 40 from fresh meat, 20 from frozen foods, 16 from fresh vegetables and fruits, and 8 from processed soybean products. Of these 142 isolates, eight isolates (5.6%, 8/142) harbored the *mecA* gene and were resistant to oxacillin, thus were MRSA. Seven of these were isolated from raw milk, the other one from frozen food (Table 1).

3.2. Multilocus sequence typing

The 142 *S. aureus* isolates were examined by MLST. As shown in Table 2, a total of 26 sequence types (STs) were revealed, which were further grouped into 16 clonal complexes (CCs) including 2 new singletons (ST2634 and ST2635). Four STs (ST2632-ST2635) were submitted as new registrations to the MLST database, because new alleles were identified in the four STs. CC188, represented solely by ST188, was the most prevalent genotype (28.9%, 41/142), followed by CC7 (21.8%, 31/142), and CC398 (9.9%, 14/142). CC5 was the most diverse clonal complex, consisting of ST5, ST512, ST965, and ST2633. Furthermore, the eight MRSA isolates were found in CC5 (ST965, 3), CC9 (ST9, 2), CC59 (ST59, 2), and CC88 (ST88, 1) clonal lineages (Table 2).

The clonal lineages of *S. aureus* isolates were further analyzed based on the food categories. As shown in Fig. 1, CC188 was the only common genotype among isolates from all five different food categories. In contrast, CC72 was only identified in *S. aureus* isolates from meat, whereas CC9 and CC59 were found in *S. aureus* isolates from milk. Interestingly, CC398 was a prevalent clone in isolates from raw milk and veg/fruits.

3.3. spa typing

The 142 *S. aureus* isolates were grouped into 34 *spa* types, including 4 newly identified *spa* types (t10761, t10777, t10793, and t10798) (Table 2). The most prevalent *spa* type was t189 (28.9%, 41/142), which mainly corresponded to isolates of ST188. Based on MLST, all five isolates within the CC88 had an identical sequence type (ST88). However, each of them carried a unique *spa* type (Table 2, CC88). This apparent superior discriminating power of *spa* typing had some exceptions. Some isolates had the identical *spa* type, but had differing STs (t441-ST50, t441-ST59, and t441-ST88) (Table 2).

Table 2
The genotypes of *S. aureus* isolates determined by three molecular typing methods.[a]

Clonal complex	MLST type	*spa* type	*agr* group[b]
CC1 (8)	ST1 (6)	t286 (3); t127 (3)	III
	ST1920 (2)	t286 (2)	
CC5 (9)	ST5 (4)	t002 (2); t548 (1); t954 (1)	II
	ST512 (1)	t548 (1)	
	ST965 (3)[c]	t062 (3)	
	ST2633 (1)	t002 (1)	
CC6 (2)	ST6 (2)	t701 (2)	I
CC7 (31)	ST7 (29)	t091 (24); t796 (1); t10798 (3); t3297 (1)	I
	ST943 (1)	t091 (1)	
	ST2632 (1)	t091 (1)	
CC9 (2)	ST9 (2)[c]	t899 (2)	II
CC15 (3)	ST15 (2)	t084 (2)	II
	ST582 (1)	t084(1)	
CC20 (5)	ST20 (2)	t164 (2)	I
	ST1281 (3)	t164 (3)	
CC50 (3)	ST50 (3)	t441 (1); t518 (1); t086 (1)	
CC59 (6)	ST59 (5)[c]	t437 (3); t441 (1); t10761 (1)	I
	ST2138 (1)	t10761 (1)	
CC72 (2)	ST72 (2)	t148 (2)	I
CC88 (5)	ST88 (5)[c]	t325 (1); t441 (1); t10777 (1); t10793 (1)	III
		t1376 (1)	IV
CC97 (9)	ST97 (7)	t267 (5); t359 (1); t1965 (1)	I
	ST464 (2)	t3297 (2)	–
CC188 (41)	ST188 (41)	t189 (40); t126 (1)	I
CC398 (14)	ST398 (14)	t034 (7); t1451 (1); t571 (3)	I
		t571(3)	variation I
CC2634 (1)	ST2634 (1)	t011 (1)	I
CC2635 (1)	ST2635 (1)	t189 (1)	I

[a] The number in parentheses represents the number of isolates in the corresponding genotype.
[b] – represents no *agr* locus was detected.
[c] The STs represents the clonal lineages of MRSA detected in this study.

3.4. agr genotyping

The distribution of *agr* alleles among the 142 food isolates is provided in Table 3. By multiplex PCR, the *agr* alleles were successfully identified in 130 isolates. The *agr* I was predominant, representing 78.5% (102/130) of the isolates and was the prevailing *agr* type regardless of the food source of *S. aureus* isolates. Twelve isolates repeatedly yielded negative results by multiplex PCR, thus their *agr* types were determined by the *Sca*I-RFLP method (Table 3). Based on *Sca*I patterns, five, one, and one isolate were grouped to *agr* I, *agr* II, and *agr* IV, respectively, whereas three isolates carried an *agr* variant. Furthermore, three isolates belonging to CC398-t571 yielded a larger PCR product (about 4 kb). Further sequence analyses revealed that the larger PCR amplicons were due to the insertion of a gene encoding a transposase between *agrD* and *agrC*. Based on the *agr* sequence, these three isolates were grouped as a variation of *agr* I. Two isolates belonging to CC97-t3297 were negative in both multiplex PCR and *Sca*I-RFLP analysis (Table 3).

3.5. Toxin gene profiles of S. aureus isolates

As shown in Table 4, there were 103 (72.5%) and 71 (50.0%) isolates carrying one toxin gene and more than one toxin genes, respectively.

Table 1
Prevalence of *S. aureus* in raw and processed food commodities in Shanghai.

Source	No. of samples	No. (%) of samples positive for *S. aureus*	No. of *S. aureus* isolated [a]	No. (%) of MRSA isolates
Raw milk	248	50 (20.2)	58	7 (12.1)
Fresh meat	128	36 (28.1)	40	0
Frozen foods	61	13 (21.3)	20	1 (5)
Processed soybean products	68	6 (8.8)	8	0
Vegetables and fruits	102	12 (11.8)	16	0
Total	607	117 (19.3)	142	8 (5.6)

[a] One to two *S. aureus* strains were isolated from each positive sample.

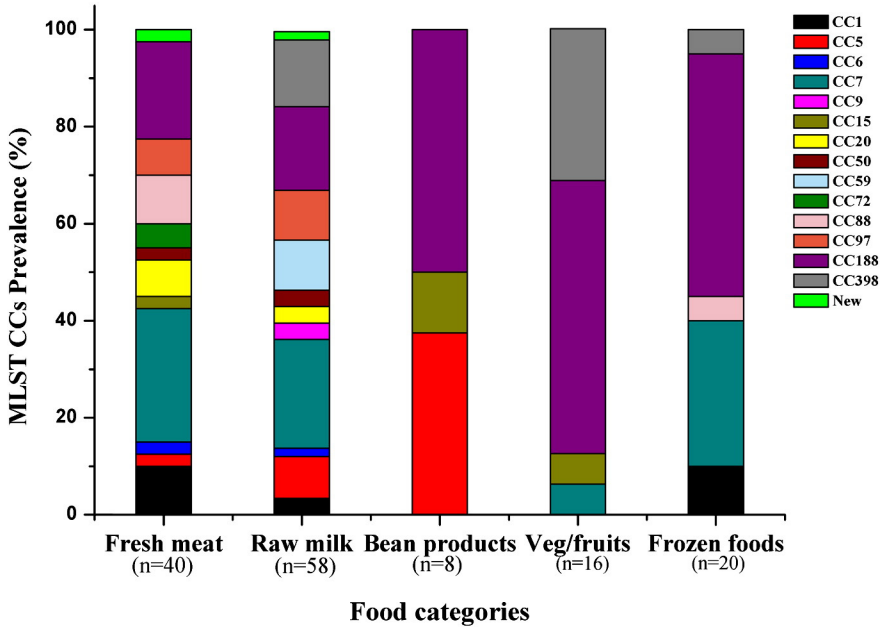

Fig. 1. Genetic diversity of *S. aureus* isolates from different food categories based on MLST. Percent prevalence of each clonal complex (CC) is expressed as the number of isolates within the same CC over the total number of *S. aureus* strains isolated from the same food category. Each color represents a unique CC. The new CCs detected in fresh meat isolates and raw milk isolates are CC2635 and CC2634, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
The *agr* types of 142 *S. aureus* isolates from different food categories.

agr group	Number of *S. aureus* isolates from various food commodities[a]					Number of *S. aureus* (total)[b]	
	Fresh meat	Raw milk	Soybean products	Veg/fruits	Frozen foods	PCR-based typing	*Sca*I RFLP
I	27 (67.5%)	47 (81.0%)	4 (50%)	12 (75%)	17 (85%)	102 (78.5%)	5
II	2 (5%)	7 (12.1%)	4 (50%)	1 (6.25%)	–	13 (10.0%)	1
III	7 (17.5%)	2 (3.45%)	–	–	3 (15%)	12 (9.23%)	–
IV	2 (5%)	2 (3.45%)	–	–	–	3 (2.31%)	1
agr variation	–	–	–	3 (18.75%)	–	–	3
agr (negative)	2 (5%)	–	–	–	–	–	2
Total	40	58	8	16	20	130	12

[a,b]The number in parentheses represents the percentage of isolates in the corresponding genotype.

It was determined in this study that the 142 *S. aureus* isolates displayed 52 different toxin gene profiles, with one to 12 toxin genes per isolate in different combinations (Table S2, Supporting information). The most prevalent toxin gene profile was the single enterotoxin gene, *sep* (20.4%, 21/103), which was found among CC7, CC188, CC398, CC97, and CC88. Different toxin gene profiles were identified among the *S. aureus* lineages (Table S2), suggesting horizontal transfer of toxin genes among *S. aureus* isolates. For example, four CC59 isolates harbored between five and eight toxin genes each, whereas the other two CC59 isolates were negative for any of the 25 toxin genes tested

Table 4
The toxin gene number of *S. aureus* isolates from different food categories.

Number of the toxin gene per strain (*n*)	Number of *S. aureus* isolates[a]					Total number of isolates (142)[b]
	Fresh meat (40)	Raw milk (58)	Soybean products (8)	Veg/fruits (16)	Frozen foods (20)	
12	0 (0)	1 (1.7%)	1 (12.5%)	0	0	2 (1.4%)
11	0 (0)	0 (0)	0 (0)	0	0	0
10	0 (0)	1 (1.7%)	1 (12.5%)	0	0	2 (1.4%)
9	0 (0)	1 (1.7%)	1 (12.5%)	0	0	2 (1.4%)
8	1 (2.5%)	3 (5.2%)	0 (0)	0	0	4 (2.8%)
7	1 (2.5%)	4 (6.9%)	0 (0)	0	0	5 (3.5%)
6	5 (12.5%)	3 (5.2%)	0 (0)	0	0	8 (5.6%)
5	0 (0)	4 (6.9%)	0 (0)	0	0	4 (2.8%)
4	2 (5.0%)	7 (12.1%)	0 (0)	0	0	9 (6.3%)
3	3 (7.5%)	11 (19.0%)	0 (0)	0	0	14 (9.9%)
2	9 (22.5%)	9 (15.5%)	1 (12.5%)	0	2 (10.0%)	21 (14.8%)
1	11 (27.5%)	8 (13.8%)	0 (0)	5 (31.3%)	8 (40.0%)	32 (22.5%)
0	8 (20.0%)	6 (10.3%)	4 (50.0%)	11 (68.7%)	10 (50.0%)	39 (27.5%)

[a] The number in parentheses represents the percentage of isolates with the corresponding number of toxin genes for all *S. aureus* isolates of the same food category.
[b] The number in parentheses represents the percentage of isolates with the corresponding number of toxin genes for all *S. aureus* isolates.

in this study. Some enterotoxin genes are known to be grouped either as a gene cluster or organized as an operon. In this study, the most prevalent toxin gene cluster was *egc* (20/142), which could be found among CC5, CC9, CC20, CC50, and CC72. The combination of *seb-sek-seq* was found in four CC59 isolates. The gene cluster *sec-sel* was found in isolates that belonged to CC1, CC5, CC7, CC59, CC188, and CC398. The gene cluster *sed-sej-ser* was found in isolates that belonged to CC5 and CC97. Taken all together, these data suggested that there was no direct association between toxin gene profile and the genotype in these *S. aureus* isolates.

The average toxin gene number in each clonal complex was also examined, and a higher average number of toxin genes (>5) was found in the isolates of CC5, CC9, CC20, CC50, and CC72 clonal lineages compared to those belonging to other CCs (Fig. 2). Consistently, isolates belonging to *agr* II ($n = 14$) or *agr* IV ($n = 4$) were associated with a high average number of toxin genes (averaging 6.8 for *agr* II, and 5.8 for *agr* IV), whereas isolates belonging to *agr* I ($n = 110$) or *agr* III ($n = 12$) were associated with a lower average number of toxin genes (averaging 1.9 for *agr* I, and 1.8 for *agr* III) (Fig. 2).

3.6. Distribution of toxin genes

The presence of 25 toxin genes in the 142 *S. aureus* isolates is shown in Fig. 3. The most frequent toxin gene was *sep* (43.7%). Notably, the abundance of classic SE genes, *sea* (5.6%), *seb* (3.5%), *sec* (9.9%), and *sed* (2.8%), often associated with staphylococcal food poisoning, was relatively lower than many recently described SEs or SEls. Among the 142 isolates, *see*, *ses*, *set*, *tsst1*, *etb*, and *etd* were not found in any isolate, whereas *eta* was only detected in one isolate (Fig. 3). PVL-encoding genes were found in 30 (21.1%) of the *S. aureus* isolates, among which, three isolates were MRSA. Furthermore, 14 of the 16 identified CCs were found among PVL-positive isolates; only CC6 and CC9 were excluded (Table S2). Moreover, it was found that three MSSA-CC398 isolates were PVL-positive.

The distribution of toxin genes among *S. aureus* isolates was further analyzed based on the food categories. The results showed that the incidence of *sec*, *sed*, *seg*, *sei*, *sej*, *sel*, *sem*, *sen*, *seo*, *ser*, and *seu*, was significantly different among the isolates from different food categories ($p < 0.05$) (Fig. 3). It was found that the four classic enterotoxin genes (*sea*, *seb*, *sec*, and *sed*) were found in isolates obtained from fresh meat, raw milk, and processed soybean products. Two *S. aureus* isolates, one from raw milk and the other from processed soybean products, carried 12 toxin genes each (Table 4). The percentage of toxigenic *S. aureus* isolates was higher for those isolates from meat (80%) or raw milk (89.7%) than any other isolates recovered from processed soybean products (50%), fresh vegetables/fruits (31.3%), or frozen foods (50%) (Table 4).

4. Discussion

The genotypes of 142 *S. aureus* isolates obtained from raw and processed food commodities in Shanghai were characterized by three molecular typing methods. It appeared that *spa* typing had a higher resolution than MLST since, for the majority of STs, more than one *spa* type was detected in this study, which is consistent with a previous study by Hata et al. (2010). Variation in the *agr* locus due to an insertion mutation was reported previously (Cafiso et al., 2007). In our study, we identified three isolates belonging to CC398 carrying an insertion mutation in their *agr* loci. Furthermore, we found the deletion of *agr* in two *S. aureus* isolates belonging to CC97 (negative for both PCR and RFLP methods), suggesting a potential limitation in utilizing this locus for molecular typing of *S. aureus* isolates. It is well known that the *agr* system controls the production of virulence factors. Montanaro et al. (2010) found that different *agr* groups exhibited distinct patterns of virulence genes. Our study showed that isolates belonging to *agr* II and *agr* IV had a higher incidence of toxin genes than those belonging to *agr* I and *agr* III, suggesting that *agr* profiles may be associated with the virulence potential of *S. aureus* (Cheung et al., 2011; Sugiyama et al., 2009).

Genotyping analysis of these 142 isolates revealed that clinical *S. aureus* clones as well as livestock-associated lineages are widespread in raw or processed food purchased in Shanghai. Among the 16 CCs identified in our study, three clonal complexes, CC5, CC7, and CC188, accounted for 57.0% of the total isolates examined. Interestingly, these three clonal complexes were prevailing in clinical isolates associated with bacteremia in China (He et al., 2013), suggesting food as a potential environmental source of *S. aureus* isolates that have an important clinical relevance. It was reported that CC97 is the dominant lineage from bovine milk in Japan (Hata et al., 2010). In our study, the isolates

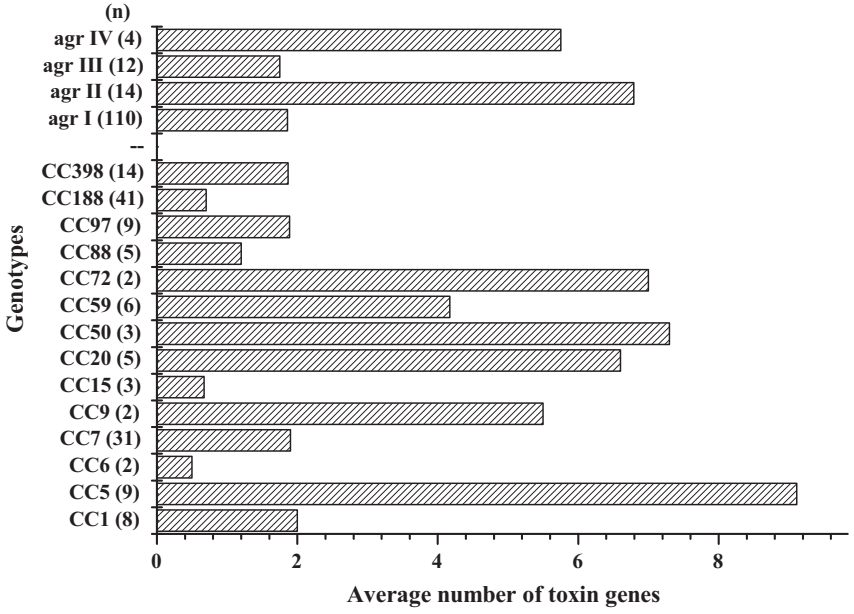

Fig. 2. The toxin gene content of *S. aureus* isolates based on MLST and *agr* genotyping. The average number of toxin genes was calculated based on the total number of toxin genes detected in each clonal complex (CC) or *agr* group divided by the total number of isolates in each CC or *agr* group. The number in parentheses on the Y-axis represents the total number of isolates in the corresponding CC or *agr* group. CC2634 and CC2635 were not included since there was only one isolate in each CC.

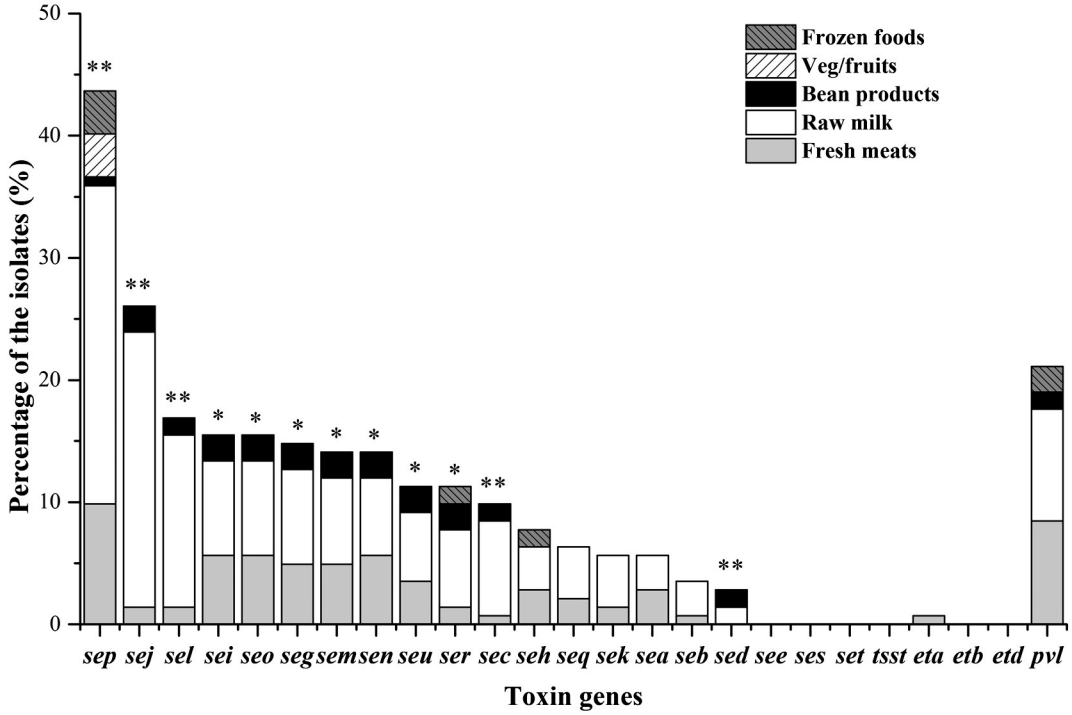

Fig. 3. The distribution of 25 toxin genes in *S. aureus* isolates from food. The percentage of strains that were positive for each toxin gene was calculated based on the origin of each food isolate. Pearson's chi-square test (two-tailed) was used to test the difference in the virulence determinants distribution among isolates from different food sources and run based on multiple group comparison of different food sources. * demonstrates that the distribution of toxin genes was statistically significantly different in isolates from different food sources ($p < 0.05$); ** demonstrates that the distribution of toxin genes was extremely significantly different in isolates from different food sources ($p < 0.001$).

belonging to CC97 were found not only in raw milk but also in fresh beef, which further implies that CC97 is associated with cattle. The eight MRSA isolates identified in our study belonged to four clonal complexes (CC5, CC9, CC59, and CC88) (Table 2). MRSA CC5 and MRSA CC88 are the prevalent clones among the HA-MRSA (Otto, 2012); and MRSA CC59 is the prevalent clone among the CA-MRSA in Asia (Li et al., 2013). The presence of these clonal lineages in food samples suggests that humans contribute largely to the food contamination with MRSA, which are likely to be introduced into the different food categories during processing/preparation by food workers. MRSA CC9 is the prevalent clone of LA-MRSA in Asian countries (Cui et al., 2009). To date the most prevalent clone of LA-MRSA in Europe and Northern America is CC398, which is rarely reported in Asia (Graveland et al., 2011). Price et al. reported that LA-MRSA CC398 appears to have originated from human MSSA-CC398, which acquired methicillin resistance in livestock (Price et al., 2012). Further analysis showed that LA-MRSA CC398 possessed many different staphylococcal cassette chromosome *mec* element (SCC*mec*) types, which suggests promiscuous dissemination of SCC*mec* cassette among strains of CC398 (Price et al., 2012; van Belkum et al., 2008). Our study revealed that MSSA-CC398 was a common clone in raw milk and meats from Shanghai. Therefore, antimicrobial selection associated with food animal production raises the potential for MSSA-CC398 in food to acquire the SCC*mec* cassette.

Clones of ST1, ST5, ST6, ST9, ST30, ST188, and ST943 are known to be associated with staphylococcal food poisoning in East Asia (Cha et al., 2006; Yan et al., 2012). Isolates with each of these genotypes were found in all five food categories investigated. However, only the isolates obtained from raw milk, fresh meat, and processed soybean products were positive for the classic SE genes that contribute significantly to staphylococcal food poisoning. Therefore, the initial amount of *S. aureus* together with the storage condition of milk, fresh meat, and processed soybean products, should be under strict control to avoid the production of SEs. The presence of toxigenic *S. aureus* strains in these foods identifies the potential sources of staphylococcal food poisoning, which is valuable information for the food industry and public health agencies in Shanghai, China. Genes encoding new types of SEs or SEls were more frequently identified than the classical SE genes in this study, implying emergence of these "new" toxigenic strains in Shanghai, as has been reported for other parts of the world (Aydin et al., 2011; Bania et al., 2006). It was reported recently by Omoe et al. (2013) that some of the new SEs, such as SElK, SElL, SElM, SElN, SElO, SElP, and SElQ, could induce emetic reactions in monkeys, suggesting a role of these new SEs in staphylococcal food poisoning.

ETA, ETB, and ETD are main human active exfoliative toxins. In our study, only one isolate from chicken was positive for the *eta* gene, suggesting a low frequency of *eta* in *S. aureus* food isolates examined in this study. A low incidence of exfoliative toxin-encoding genes was reported previously for milk samples and ready-to-eat food (Aydin et al., 2011; Karahan et al., 2009). Furthermore, none of the 142 isolates examined in our study was positive for the *tsst1* gene, which has been found in clinical *S. aureus* isolates associated with important clinical symptoms (Xie et al., 2011). Notably, a high prevalence of PVL-encoding genes was found in genetically diverse MSSA isolates from Shanghai in this study. Previous studies have reported that PVL-positive MSSA strains showed diverse genetic backgrounds, indicating that the bacteriophage carrying the PVL genes can be spread easily (Chini et al., 2006). The majority of PVL-positive CC398 isolates described so far have been obtained from humans (van Belkum et al., 2008; Yu et al., 2008), but few have been found in livestock. In this study, three MSSA-CC398 isolates, from milk and meat, were PVL-positive. Phylogenetic analysis has shown that LA-MRSA CC398 appears to originate from human MSSA-CC398 (Price et al., 2012). Therefore, LA-MRSA CC398 may acquire the PVL genes through horizontal gene transfer via bacteriophage. Considering that CC398 is by far the most prevalent LA-MRSA worldwide, the finding of PVL-encoding genes in CC398 food isolates poses a potential risks to public health.

Although no direct association between *S. aureus* genotype and toxin gene profile was found in 142 isolates examined in our study, certain genotypes such as CC5, CC9, CC20, CC50 and CC72 were found to carry more toxin genes (>5) than other CCs. The association of CC5, CC20,

and CC72 with a high content of toxin genes was reported previously (Varshney et al., 2009). In our study, CC5 was identified in isolates obtained from fresh meat, raw milk, or processed soybean products; *S. aureus* strains of CC20 and CC50 were obtained from fresh meat or raw milk; CC9 *S. aureus* strains were isolated from milk, whereas strains of CC72 were isolated from meat. The association of toxin gene rich-genotypes with specific food matrices may explain the higher incidence of toxin genes in milk or meat isolates compared to those from frozen food or fresh vegetables and fruits observed in our study.

In summary, our study demonstrated genetic diversity and virulence potential of *S. aureus* isolates obtained from different food categories at retail in Shanghai. A large variation in genetic diversity as well as toxin gene content was observed for *S. aureus* isolates from raw and processed food commodities. It was revealed that both, raw milk and fresh meat were the major sources of isolates containing multiple toxin genes. However, as the majority of isolates were obtained from raw milk and fresh meat, there is a possible bias on the genetic diversity of isolates obtained from different food categories, which still needs to be further investigated. Furthermore, *S. aureus* isolates from food that are genetically highly related, displayed diverse toxin gene profiles, implying a role of horizontal gene transfer in the emergence of toxigenic *S. aureus* isolates (Alibayov et al., 2014; Malachowa and DeLeo, 2010).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2014.11.020.

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