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Original Article

High Prevalence and Properties of Enterotoxin-Producing *Staphylococcus aureus* ST5 Strains of Food Sources in China

Yanzi Chang,[1] Hong Gao,[2] Zhenhua Zhu,[3] Shuo Ye,[2] Yuanbin Yang,[2] Xuanyi Shen,[2] Danyang Zhang,[2] and Qifa Song[2]

Abstract

Staphylococcus aureus with the ability of staphylococcal enterotoxins (SEs) production is one of the most common causes of bacterial foodborne outbreaks worldwide. In our study, 336 *S. aureus* isolates were recovered from 3476 food samples during 2010–2014. A total of 86 *S. aureus* isolates were proved to be enterotoxin-producing strains with PCR and enzyme-linked immunosorbent assay. In the 86 isolates, 20 STs were identified using multilocus sequence typing (MLST) and 20 isolates were typed as sequence type 5 (ST5), which was the most prevalent ST using MLST. There were six SE profiles and high carrier rates of *sec* (50%) and *sed* (75%) genes in the 20 *S. aureus* ST5 isolates. Additionally, 8 antibiotic resistance patterns were observed, and 10 multidrug-resistant isolates (50%) and 4 methicillin-resistant *S. aureus* isolates were identified. Our findings illustrate high prevalence of *S. aureus* ST5 isolates from food sources and diversity in SE profiles and antibiotic resistance patterns. These results indicate that great difference in the ability of obtaining SE production and antimicrobial resistance may exist between different genetic lineages of *S. aureus* strains.

Introduction

Staphylococcal food poisoning (SFP) is one of the most common forms of bacterial foodborne outbreaks worldwide. *Staphylococcus* spp. and their bacterial toxins have been reported to be the fourth most common causative agent in foodborne outbreaks in Europe in 2011 (Eurosurveillance editorial team, 2013). SFP occurs after ingestion of food contaminated with enterotoxin-producing *Staphylococcus aureus* by improper handling and storage, which allows growth of bacteria and resulting toxin production. Certain *S. aureus* strains possess the ability to produce various staphylococcal enterotoxins (SEs). SEs are heat-stable exotoxins, which can cause various symptoms, including diarrhea and vomiting. Classical SEs are classified using serological method into five types: SEA, SEB, SEC, SED, and SEE (Dinges *et al.*, 2000). Previous studies have reported the various SE profiles and different production levels in isolates from specific food sources (Wallin-Carlquist *et al.*, 2010; Tang *et al.*, 2011).

To analyze the genetic diversity of *S. aureus* isolates and the relatedness between strains, multilocus sequence typing (MLST) has been used for its high reproducibility and discriminatory capability to compare results between laboratories (Smith *et al.*, 2005; Sasaki *et al.*, 2012). MLST is a useful tool to find correlation between diseases and particular clones (Enany *et al.*, 2007; Sharma *et al.*, 2014). MLST also helps to determine clonal origin and find particular clones with unique traits, for example, ST398 strains which has gained particular attention during recent years because of its association with pigs and its ability to colonize pig farmers and other people in close contact with pigs (Argudin *et al.*, 2011), and ST80 strains showing unique antibiotic resistance profile between districts (Enany *et al.*, 2010).

During 2010–2014, we identified 86 enterotoxin-producing *S. aureus* strains recovered from 3476 food samples. Interestingly, up to 20 *S. aureus* isolates were typed as ST5, which was the most prevalent ST in 86 isolates. The aim of this study was to further characterize the 20 *S. aureus* ST5 strains based on the SE expression and profiles, antibiotic resistance patterns and methicillin-resistant *S. aureus* (MRSA), and distribution.

Materials and Methods

Sampling and S. aureus *identification*

A total of 336 (9.7%) *S. aureus* strains were recovered from 1536 milk and dairy samples ($n = 106$) and 1940 meat

[1]Ningbo Medical Treatment Center Lihuili Hospital, Ningbo, China.
[2]Department of Microbiology, Ningbo Municipal Centre for Disease Control and Prevention, Ningbo, China.
[3]Xiangshan Entry–Exit Inspection and Quarantine Bureau, Ningbo, China.

samples ($n=230$) in Ningbo city (mid-east China) during 2010–2014. Seven types of food were included: cheese cake ($n=710$, 82 isolates), ice cream ($n=360$, 11 isolates), liquid milk ($n=310$, 5 isolates), milk powder ($n=156$, 7 isolates), beef ($n=415$, 74 isolates), pork ($n=695$, 67 isolates), and chicken ($n=830$, 90 isolates). All samples were transferred to the microbiology laboratory under required preservation conditions and processed within their expiration date (or within 2 h for samples without expiration date) to test for the presence of *S. aureus*. Isolation and identification of *S. aureus* were conducted by enrichment and sequential plating onto selective plates as described previously (de Boer *et al.*, 2009). Briefly, 25 mL or 25 g of each sample was mixed with 225 mL of brain-heart infusion (BHI) broth with 6.5% NaCl (Beijing Land Bridge Technology Ltd.) and incubated at 37°C overnight. The incubated broth was streaked onto chromogenic plates (CHROMagar) to isolate presumptive isolates of *S. aureus*. Then, the presumptive isolates were identified as *S. aureus* following standard laboratory techniques, including Gram stain, coagulase assay, and catalytic reactions using VITEK 2 GP card (bioMérieux). One colony of identified *S. aureus* was selected for each sample for further analysis.

PCR assay for detection of enterotoxin genes and mecA *gene*

The DNA extraction was performed using the Bacterial Genomic DNA Extraction Kits (TaKaRa MiniBEST, Ver.3.0) following the protocol. Primers for five classical enterotoxin genes and *mecA* gene were used (Table 1). *S. aureus* ATCC12600 was used as a negative control in all PCRs. *S. aureus* ATCC13565 was used as a positive control for *sea* and *sed*, *S. aureus* ATCC19095 for *sec*, *S. aureus* ATCC14458 for *seb*, and *S. aureus* ATCC27664 for *see*. All control strains were obtained from the China Center of Industrial Culture Collection. PCR was conducted using the Commercial PCR Kit (TaKaRa) in 25 μL volume containing 2.5 μL of buffer (10×, Mg^{2+} plus), 1.5 μL of dNTP (2.5 mM), 0.5 μL of each primer set (10 μM), 1.0 μL of Taq DNA polymerase (1 U/μL), and 2.0 μL of template DNA. The PCR cycling consisted of an initial denaturation at 95°C for 3 min, 30 cycles of 94°C for 45 s, 52°C for 45 s, and 72°C for 60 s, and a final extension at 72°C for 5 min. The amplicons were separated with electrophoresis in 1.5% agarose gel and visualized under UV light after staining with Ethidium Bromide to verify the expected size of PCR products.

Multilocus sequence typing

DNA template was obtained as mentioned above. MLST was conducted using seven sets of oligonucleotide primers for each of the seven relevant housekeeping MLST genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL*) listed on the *S. aureus* MLST website (http://saureus.mlst.net/, updated on 23/11/2015). The allelic profile of a *S. aureus* isolate appearing as an array of seven allele numbers was achieved by sequencing internal fragments of seven housekeeping genes (Invitrogen Company) and the STs were assigned using the same website.

*Staphylococcal protein A (*spa*) typing*

PCR was conducted using oligonucleotide primers for *spa* typing and DNA sequencing was performed (Invitrogen Company) (Shopsin *et al.*, 1999). Briefly, the repeat-containing region of *spa* gene was amplified by PCR followed by DNA sequencing of the amplicons. The *spa* typing analysis was carried out using eGenomics software (www.egenomics.com), and Ridom *spa* types were assigned using the SpaServer website (http://spaserver2.ridom.de).

Antimicrobial susceptibility testing

The minimal inhibitory concentration (MIC) values were obtained using AST-GP67 card on VITEK2 (bioMérieux). Antimicrobial susceptibility testing (AST) results were interpreted according to the guidelines in Clinical and Laboratory Standards Institute (CLSI) document M100-S24. The following MIC interpretive criteria indicating resistance were used: penicillin (≥0.25 μg/mL), oxacillin (≥4 μg/mL), cefoxitin (≥6 μg/mL), tetracycline (≥16 μg/mL), ciprofloxacin (≥4 μg/mL), gentamicin (≥16 μg/mL), erythromycin (≥8 μg/mL), clindamycin (≥8 μg/mL), trimethoprim–sulfamethoxazole (≥4 μg/mL/76 μg/mL), tigecycline (≥2 μg/mL), linezolid (≥8 μg/mL), and vancomycin (≥16 μg/mL). *S. aureus* ATCC25923 was used as a quality control strain. Strains resistant to three or more drugs of chemically unrelated classes were defined as multidrug resistance (MDR) (Magiorakos *et al.*, 2012). Isolates resistant to

Table 1. Oligonucleotide Sequences for Polymerase Chain Reaction Amplification of Enterotoxin Genes and *mecA* Gene in *Staphylococcus aureus* Isolates

Gene	*Primer name*	*Sequence (5′→3′)*	*Amplicon size (bp)*	*Reference*
Sea	*sea-f*	CCTTTGGAAACGGTTAAAACG	127	Becker *et al.* (1998)
	sea-r	TCTGAACCTTCCCATCAAAAAC		
seb	*seb-f*	TCGCATCAAACTGACAAACG	477	Becker *et al.*, (1998)
	seb-r	GCAGGTACTCTATAAGTGCCTGC		
sec	*sec-f*	AGATTTAGCAAAGAAGTACAAAGATG	490	Becker *et al.*, (1998)
	sec-r	AAGGTGGACTTCTATCTTCACACTT		
sed	*sed-f*	GAGGTGTCACTCCACACGAA	349	Varshney *et al.* (2009)
	sed-r	TGAAGGTGCTCTGTGGATAATG		
see	*see-f*	ACCGATTGACCGAAGAAAAA	264	Varshney *et al.* (2009)
	see-r	ATTGCCCTTGAGCATCAAAC		
mecA	*mecA-f*	TTGTAGTTGTCGGGTTTGGT	489	This study
	mecA-r	TTGGAACGATGCCTATCTCA		

oxacillin and/or cefoxitin were further confirmed to be MRSA by amplification of *mecA* gene.

Enzyme-linked immunosorbent assay for detection of SEs

The detection of various SEs was done for the isolates with enterotoxin gene using Ridascreen SET Total and SET A, B, C, D, and E (R-Biopharm) following the manufacturer's manual. Briefly, *S. aureus* strains were enriched in BHI broth at 37°C overnight, then supernatant was centrifuged at $3500 \times g$ at 10°C for 5 min and filtered sterilely and 100 μL of the filtrate was used to measure the optical density (OD) of SEs following ELISA protocol. Results were judged positive when OD value was over cutoff value for the assay (cutoff value = absorbance value of negative control +0.15).

Results

Up to 86 (25.6%, 86/336) isolates were tested positive for one or more classical SE genes using PCR and further proved to be SE-producing using ELISA SET Total. Overall, 20 STs were identified by MLST in the 86 *S. aureus* isolates. The top five most prevalent STs were ST5 ($n = 20$), ST1 ($n = 16$), ST188 ($n = 10$), ST6 ($n = 9$), and ST15 ($n = 5$), accounting for 69.8% (60/86). The 20 *S. aureus* ST5 strains scattered over 5 years: 2010 ($n = 5$), 2011 ($n = 2$), 2012 ($n = 6$), 2013 ($n = 4$), and 2014 ($n = 3$), and in five types of food: beef ($n = 5$), cheese cake ($n = 5$), pork ($n = 4$), chicken ($n = 4$), and ice cream ($n = 2$) (Table 2). The 20 *S. aureus* ST5 strains were further typed using Ridom *spa* typing and divided into five *spa* types, including t548 ($n = 16$), t386 ($n = 1$), t127 ($n = 1$), t472 ($n = 1$), and t933 ($n = 1$) (Table 2).

In addition, the 20 *S. aureus* ST5 strains were proved to be SE-producing in various SE profiles using the ELISA Kit SET A, B, C, D, and E (Table 2). The following detection rates for five classical SE genes were achieved in the 20 *S. aureus* ST5 strains: *sea* (5%, 1/20), *seb* (10%, 2/20), *sec* (50%, 10/20), *sed* (75%, 15/20), *see* (0%). Of the five classical SEs, SED was most prevalent. Six SE profiles were identified in 20 strains, including SED (45%, 9/20), SEC+SED (25%, 5/20), SEC (15%, 3/20), SEA+SEC (5%, 1/10), SEB+SEC (5%, 1/20), and SEB+SED (5%, 1/20) (Table 2).

Finally, AST results for the 20 *S. aureus* ST5 strains showed different resistance to penicillin (75%), erythromycin (55%), trimethoprim–sulfamethoxazole (45%), clindamycin (35%), ciprofloxacin (20%), oxacillin (20%), cefoxitin (20%), and gentamicin (10%). Isolates resistant to teicoplanin, tetracycline, linezolid, and vancomycin were not observed. Four cefoxitin-resistant *S. aureus* strains were confirmed to be MRSA by the amplification of *mecA* gene. Ten isolates (50%) were MDR. Four isolates (20%) were not resistant to any antimicrobial tested. Overall, there were eight antibiotic resistance patterns (including sensitive pattern) (Table 2).

Discussion

Our study demonstrated high prevalence of particular *S. aureus* ST5 isolates in SE-producing *S. aureus* from food source. Various SE profiles and antibiotic resistance patterns were observed in these *S. aureus* ST5 isolates, which were recovered over 5 years and in different types of food.

To date, although new types of SEs (SEG-SElV and some SE-like superantigens) have been described (Su and Wong, 1995; Munson *et al.*, 1998), it is still unclear whether some

Table 2. *spa* Types, Antimicrobial Susceptibility Testing Patterns, and Enterotoxin Profiles of 20 *S. aureus* ST5 Strains

Strain name	*Isolation date (month/year)*	*Source*	*Ridom* spa *type*	*AST pattern*[a]	*Enterotoxin profile*	*PCR results*
Ptq6	01/2010	Beef	t548	P, E, TMP-SMX	SED	*sed*
Ptq7	01/2010	Beef	t548	P, E, TMP-SMX	SED	*sed*
Ptq14	02/2010	Cheese cake	t548	P	SEC, SED	*sec*, *sed*
Ptq15	02/2010	Pork	t548	P	SEC, SED	*sec*, *sed*
Ptq34	07/2010	Beef	t548	P, DA, E, TMP-SMX	SED	*sed*
Ptq59	03/2011	Cheese cake	t548	P, E, TMP-SMX	SEB, SED	*seb*, *sed*
Ptq68	05/2011	Pork	t548	P, DA, E, CN	SEC, SED	*sec*, *sed*
Ptq170	05/2012	Chicken	t548	P, TMP-SMX	SED	*sed*
Ptq184	07/2012	Beef	t548	P, OX, FOX, CIP, DA, E, TMP-SMX	SEB, SEC	*mecA*; *seb, sec*
Ptq185	07/2012	Chicken	t548	P, OX, FOX, CIP, DA, E, TMP-SMX	SEC	*mecA*; *sec*
Ptq188	07/2012	Cheese cake	t127		SEC, SED	*sec*, *sed*
Ptq190	10/2012	Ice cream	t548	P, OX, FOX, CIP, DA, E, TMP-SMX	SEC	*mecA*; *sec*
Ptq193	12/2012	Cheese cake	t548	P	SEC, SED	*sec*, *sed*
Ptq202	02/2013	Pork	t386	P	SED	*sed*
Ptq262	07/2013	Beef	t548		SEA, SEC	*sea*, *sec*
Ptq274	10/2013	Pork	t933	P, OX, FOX, CIP, DA, E, TMP-SMX	SEC	*mecA*; *sec*
Ptq281	12/2013	Chicken	t472		SED	*sed*
Ptq297	03/2014	Cheese cake	t548	P, DA, E, CN	SED	*sed*
Ptq307	06/2014	Ice cream	t548	E	SED	*sed*
Ptq331	12/2014	Chicken	t548		SED	*sed*

[a]Abbreviations for antimicrobials.

AST, antimicrobial susceptibility testing; CIP, ciprofloxacin; CN, gentamicin; DA, clindamycin; E, erythromycin; FOX, cefoxitin; OX, oxacillin; P, penicillin; PCR, polymerase chain reaction; TE, tetracycline; TMP-SMX, trimethoprim–sulfamethoxazole.

new SEs are responsible for food poisoning (Sakai *et al.*, 2008). Moreover, about 95% of SFP outbreaks are caused by the classical SEs, and the remaining 5% of outbreaks are associated with other identified SEs (Kokan and Bergdoll, 1987). Thus, in this study, we targeted five classical SEs to screen SE-producing *S. aureus*. All of the 20 *S. aureus* ST5 strain were proved to be able to express relevant SEs above detection limit of ELISA (i.e., 1 ng/mL).

It is noteworthy the high prevalence (23.3%) of *S. aureus* ST5 strains among 86 SE-producing *S. aureus* strains in our study. To compare the difference in the distribution between SE-positive and SE-negative strains, we randomly selected 40 strains as control strains that were negative for the five SEs and isolated from the same region during the study period. The MLST distribution of SE-negative strains was as following: ST1 (5), ST7 (4), ST59 (3), ST88 (5), ST6 (2), ST15 (1), ST188 (4), ST20 (3), ST50 (2), ST97 (3), ST398 (3), ST1046 (1), ST489 (1), ST630 (1), ST2983 (1), and ST3262 (1). No ST5 was found. This difference suggests that *S. aureus* ST5 strains tend to obtain greater ability of SE producing in view of the much higher prevalence of ST5 *S. aureus* strains among 86 SE-positive *S. aureus* strains than SE-negative strains. In other words, there may exist different potential to become enterotoxigenic for strains of certain lineages. In this study, we found difference in both SE profiles and antibiotic resistance patterns in *S. aureus* ST5 isolates with high genetic relatedness. To make molecular typing more reliable, we further conducted *spa* typing on the 20 *S. aureus* ST5 strains. Of the 20 *S. aureus* ST5 strains, 16 were typed as t548 by *spa* typing. Diversity in SE profiles and antibiotic resistance patterns were still found in these16 isolates (Table 2).

The molecular mechanisms for spreading five classical SEs (SEA-SEE) may be the cause of the SE diversity in strains with high genetic relatedness. Several mobile genetic elements, such as prophages, plasmids, and pathogenicity islands (*SaPI*), are responsible for encoding of different enterotoxins. *sea* and *see* genes are carried by a polymorphic family of temperate bacteriophages (Betley and Mekalanos, 1985). The *seb* and *sec* genes are carried on SaPI3 and SaPI4, respectively (Novick *et al.*, 2001; Novick, 2003). SaPIs are highly mobile phage-related staphylococcal pathogenicity islands that can integrate into specific sites (known as the *attC* sites) in the chromosome determined by the specificity of SaPI-encoded integrases (Schelin *et al.*, 2011). The *sed* gene is situated on a 27.6 kb penicillinase plasmid, pIB485 (Bayles and Iandolo, 1989). The elements for five classical SEs can be gained or lost in environment. Although these 20 *S. aureus* ST5 strains have high genetic relatedness, they might have obtained various genetic elements in different environments over 5 years and from different food types, which lead to the diversity in SEs. Moreover, we found that 75% and 50% of these strains harbored *sed* and *sec* genes, respectively. We considered that ST5 might be highly related with *sed* and *sec*. Our findings also illustrate different incidence of resistance for SE-producing *S. aureus* ST5 strains compared with strains of food sources in the literature for some drugs: ciprofloxacin (20% in our study vs. 2% in reported); and gentamicin (10% in our study vs. 1% in reported) (Buyukcangaz *et al.*, 2013). Especially, four MRSA strains (20%) were observed in 20 *S. aureus* ST5 strains. Likewise, all of two MRSA isolates found in 133 *S. aureus* in a previous research are ST5 (Buyukcangaz *et al.*, 2013). The high incidence of resistance for *S. aureus* ST5 strains is also reported on www.mlst.net. These resistance results reveal that ST5 strains may be more inclined to become resistant.

In conclusion, this study reveals the high prevalence of *S. aureus* ST5 strains from food sources and their diversity in SE profiles and antibiotic resistance patterns. These results indicate the great difference in the ability of obtaining SE production and antibiotic resistance between different genetic lineages of *S. aureus* strains from food.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Qifa Song, PhD
Department of Microbiology
Ningbo Municipal Centre for Disease Control and Prevention
Ningbo 315010
Zhejiang Province
China

E-mail: songqf@nbcdc.org.cn