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Distribution of food-borne *Staphylococcus aureus* enterotoxin genes

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ABSTRACT. We identified and analyzed 5 new-type enterotoxin genes, including *SEj*, *SEl*, *SEq*, *SEm*, and *SEr*, to explore the distribution of 5 enterotoxin genes in *Staphylococcus aureus* of different origins as well as their correlations and differences. We examined the distribution of the *S. aureus* enterotoxin genes and their pathogenic mechanisms. A total of 660 specimens were collected from January 2011 to December 2014, and 217 strains of *S. aureus* were isolated. The template DNA of *S. aureus* was extracted. The Primer6.0 and Oligo7 software were used to design and synthesize polymerase chain reaction primers. Amplification results were analyzed by electrophoresis, and the amplification products were recovered and sequenced. Thirty-six bacterial strains contained the *SEj* gene (16.6%), including 15, 8, 8, 4, and 1 strains in fresh meat, quick-frozen food, raw milk, human purulent tissue, and living environment, respectively. Thirty-one bacterial strains contained the *SEr* gene (14.3%), including 16, 9, and 6 strains in fresh meat, quick-frozen food, and raw milk, respectively. Twenty-one bacterial strains contained the enterotoxin *SEq* gene (9.7%), including 8, 6, 6, and 1 strains in fresh meat, quick-frozen food, raw milk, and human purulent tissue, respectively. No *SEm* and *SEl* genes were detected. Different types of foods carry different types of enterotoxins, providing a basis for quick tracing for food poisoning.

Three enterotoxin genes, *SEj*, *SEr*, and *SEq*, showed the highest carrier rate in quick-frozen food. It is imperative to improve their detection in quick-frozen food.

Key words: New-type enterotoxin gene; *Staphylococcus aureus*; Gene; Food contamination

INTRODUCTION

Staphylococcus is an important and common type of zoonotic pathogenic bacterium that is extensively distributed in nature (air, water, dust, and excrement of humans and animals) (Bergdoll, 2010). *Staphylococcus* can contaminate food during both preparation and processing (Reiser et al., 1983; Jarraud et al., 1999). *Staphylococcus aureus* is strongly pathogenic, accounting for approximately 65% of bacterial food poisoning incidents in Japan and approximately 35% in the US. This percentage is also rising in some European countries. In recent years, similar food poisoning events have also occurred in China, involving many regions and populations (Sen, 2000; Johnson et al., 1991). In recent years, food poisoning accidents have largely involved milk, meat, fish, eggs and their related products, leftover food, and public contact areas, with infection sources mainly including human and livestock pyogenic infection sites.

The pathogenicity of *S. aureus* is mainly attributable to toxins and invasive enzymes generated under specific environmental conditions. More than 20 types of toxic proteins have been reported (Hermans et al., 2001; Trautmann et al., 2002). The major toxins include staphylococcal enterotoxins (SEs), lysostaphin, exfoliative toxin, leukocidin, and toxic shock syndrome toxin-1. The main enzymes generated include coagulase, heat-stable nuclease, and staphylokinase, as well as many others. SE plays an important role in the pathogenicity of the bacteria. It has been reported that *S. aureus* food poisoning can occur when 18 μg SE are present in 100 g food. Thus, it is necessary to detect the sources of SE and its distribution.

In this study, we identified and analyzed 5 new-type enterotoxin genes, *SEj*, *SEl*, *SEq*, *SEm*, and *SEr* in 217 strains of *S. aureus* from 6 different origins preserved in the laboratory using polymerase chain reaction (PCR). We explored the distribution of the 5 enterotoxin genes in *S. aureus* of different origins as well as their correlations and differences in order to increase the understanding of staphylococcal enterotoxin genes. Our results provide a basis for examining the distribution of the *S. aureus* enterotoxin genes and their pathogenic mechanisms, as well as guide the rapid medical clinical diagnosis of food poisoning resulting from *S. aureus* and prevention of such food poisoning.

MATERIAL AND METHODS

Bacterial strains

A total of 660 specimens of bacterial strains preserved in the laboratory were collected from January 2011 to December 2014. A total of 217 strains of *S. aureus* were isolated, including 89 strains from fresh meat (pork, beef, mutton, and chicken), 42 strains from quick-frozen food, 44 strains from raw milk, 32 strains from human purulent tissue, 3 strains from bean products, and 7 strains from the living environment.

Apparatus

In this study, we used a 5417R high-speed refrigerated centrifuge (Shanghai Anting Scientific Instrument Factory, Shanghai, China), HH.SY11-Ni Electronic Thermostat Water Bath (Beijing Guangming Medical Instrument Factory, Beijing, China), TGradient Thermocycler96 PCR amplifier (Biometra, Göttingen, Germany), DYCp-32 electrophoresis tank (Liuyi Instrument Plant, Beijing, China), WD-9403C ultraviolet analyzer (Liuyi Instrument Plant), DYY-8B voltage-stable and current-stable electrophoresis apparatus (Liuyi Instrument Plant), and BTS-20M gel imaging system (UVItec, Ltd., Cambridge, UK).

Reagents and culture media

Lysozyme was from Whatman plc (Little Chalfont, UK), acetone was from Beijing Chemical Reagents Co., Ltd. (Beijing, China), agarose was obtained from Yitong Biological Instruments Co., Ltd. (Shanghai, China), and nutrient broth prepared in accordance with GB4789.28-94.

Extraction of DNA in *S. aureus* template

First, 1.5 mL bacterial suspension was collected in a centrifuge tube and centrifuged at 11,000 *g* for 2 min. The bacteria were collected, precipitated, and mixed with 200 mL lysozyme, followed by mixing with 2 mL proteinase K and heating in a water bath at 37°C for 2 h. The reaction solution was incubated for 20 min at room temperature after the addition of 200 mL sodium dodecyl sulfate-Tris to terminate the reaction. The supernatant was collected and centrifuged at 12,000 rpm for 5 min after the addition of 600 mL 5 M NaCl. The reaction solution was reversed for 10 min and centrifuged at 12,000 rpm for 0.5 h after the addition of 400 mL pre-cooled phenol-chloroform. The supernatant, which represented the aqueous phase, was collected and mixed with 400 mL pre-cooled phenol-chloroform. These steps were repeated once. The mixture was incubated for 2 h and centrifuged at 4°C for 10 min at 12,000 rpm after the addition of 0.1X volume of sodium acetate and 2X volume -20°C absolute ethyl alcohol. The supernatant was discarded. The tube was allowed to sit for 1 min on a piece of absorbent paper and dried at 37°C for 1 min. Finally, 50 µL sterilized water was added (Wilden et al., 2014).

Design of primers

Based on published enterotoxin gene sequences, the Primer6.0 and Oligo7 primer design softwares were used to design and synthesize PCR primers (Li et al., 2014). Table 1 shows the primer sequences.

Table 1. PCR primers.

Primer	Sequence (5'→3')	Product size (bp)
SEj-F	CACCAGAACTGTTGTTCTGCTAG	114
SEj-R	CTGAATTTTACCATCAAAGGTAC	
SEr-F	TCCCATTCCTTATTTAGAATACA	440
SEr-R	GGATATTCCAAACACATCTGAC	
SEm-F	AGTTTGTGTAAGAAGTCAAGTGTAGA	178
SEm-R	ATCTTTAAATTCAGCAGATATTCCATCTAA	
SEl-F	TGGACATAACGGCACTAAAA	145
SEl-R	TTGGTARCCCATCATCTCCT	
SEq-F	ATACCTATTAATCTCTGGGTCAATG	222
SEq-R	AATGGAAAGTAATTTTTCCTTTG	

PCR

The PCR was conducted as described by Letertre et al. (2003a). The total reaction volume was 25 µL and included the following: 18.25 µL water, 2.5 µL 10X PCR buffer, 1.5 µL 25 mM Mg^{2+}, 0.5 µL 1 mM dNTPs, 0.5 µL 20 µM of each primer, 0.25 µL 5 U/µL Taq enzyme, and 1 µL template DNA.

The PCR conditions were as follows: pre-denaturation at 94°C for 40 s, annealing at 52°C for 40 s, and extension at 72°C for 1 min for a total of 30 cycles, followed by a final extension for 10 min at 72°C. This procedure was used for all genes except *SEj*, for which the annealing temperature was 55°C.

Electrophoresis

The PCR products were subjected to 1% agarose gel electrophoresis. The gel was imaged using a gel-imaging system after electrophoresis. PCR amplification results were analyzed based on comparison with DNA of standard size.

Sequencing

The PCR products were collected and sequenced. The sequencing results were searched in NCBI to examine the distribution of the 3 enterotoxin genes in wild-type *S. aureus* and to verify the relationship between these genes and bacterial strains.

RESULTS

Bacterial strains containing different enterotoxin genes were obtained from the 217 strains of *S. aureus* from 6 different origins, which were extracted and preserved in the laboratory after agarose gel electrophoresis and sequencing (Table 2). Figure 1 shows the detection rates of *S. aureus* in various types of specimens. Fresh meat products showed the highest detection rate of *S. aureus* (49.4%) among the samples. Mutton showed the highest detection rate among the 5 types of fresh meat examined. The detection rates of enterotoxin genes from *S. aureus* in meat products, quick-frozen food, and other food types were higher than in unprocessed food.

Table 2. Detection of 5 enterotoxin genes.

Bacterial strains	Number of specimens	Number of *S. aureus* strains detected	Detection rate (%)	*SEj*	Detection rate (%)	*SEr*	Detection rate (%)	*SEq*	Detection rate (%)	*SEm*	*SEl*
Fresh meat	180	89	49.4	15	16.9	16	18.0	8	9.0	0	0
Quick-frozen food	150	42	28.0	8	19.0	9	21.4	6	14.3	0	0
Raw milk	120	44	36.7	8	18.2	6	13.6	6	13.6	0	0
Human purulent tissue	100	32	32.0	4	12.5	0	0	1	3.1	0	0
Living environment	60	7	11.7	1	14.3	0	0	0	0	0	0
Bean products	50	3	6.0	0	0	0	0	0	0	0	0
Total	660	217		36		31		21		0	0
Detection rate (%)		32.9		16.6		14.3		9.7		0	0

PCR identification of *SEj*

A positive result was observed on gel electrophoresis for *SEj* after PCR, as indicated in Figure 1. The length of the amplified fragment was 114 base pairs. The results revealed that 36 bacterial strains contained the *SEj* gene, with a detection rate of 16.6%, including 15 strains

in fresh meat and meat products, 8 strains in quick-frozen food, 8 strains in raw milk, 4 strains in human purulent tissue, and 1 strains in living environment. The detection rate of *S. aureus* containing j-type enterotoxins was the highest in quick-frozen food. No *S. aureus* containing j-type enterotoxins was detected in bean products. The detection rate of A-type enterotoxins was highest in quick-frozen food (19.0%), followed by in raw milk.

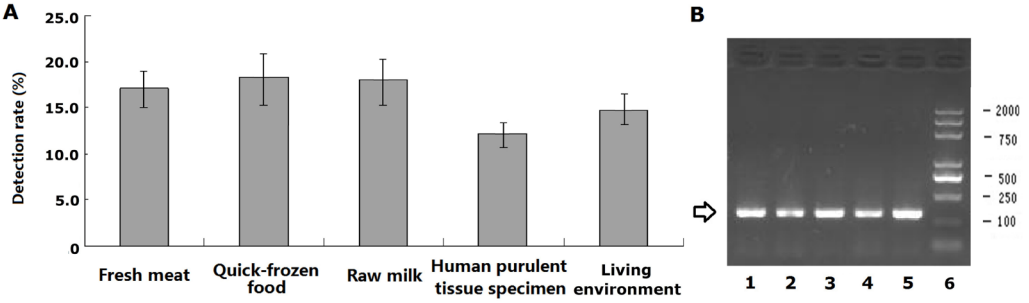

Figure 1. A. PCR amplification result of *SEj*. The detection rate in Quick-frozen food was the highest. **B.** The length of the amplification fragment was 114 bp. *Lanes 1-4* = *SEj* gene. *Lane 5* = standard strain of *S. aureus*. *Lane 6* = DL2000 DNA marker.

PCR identification for *SEr*

SEr encodes for an uncommon enterotoxin gene in *S. aureus*. The enterotoxin shows strong thermostability. The detection rate of *SEr* was 14.3%. A positive result was observed for specific amplification of *SEr* by PCR, as indicated in Figure 2. The length of the amplified fragment was 440 base pairs. Thirty-one bacterial strains contained the SEr gene, including 16 in fresh meat and meat products, 9 in quick-frozen food, and 6 in raw milk. No bacterial strains were detected in other specimens. The detection rate was highest in quick-frozen food (21.4%).

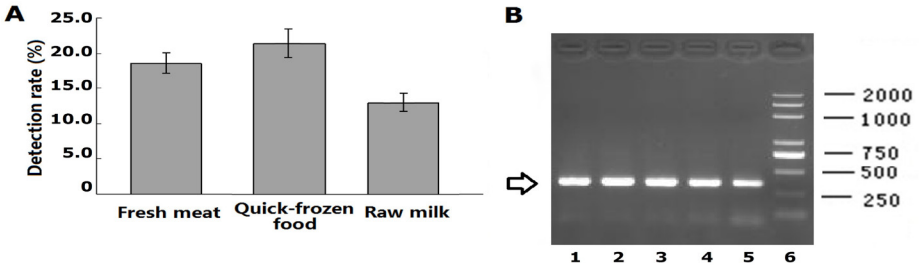

Figure 2. A. PCR amplification result of *SEr*. The detection rate in Quick-frozen was the highest. **B.** The length of the amplification fragment was 440 bp. *Lanes 1-4* = *SEr* gene. *Lane 5* = standard strain of *Staphylococcus aureus*. *Lane 6* = DL2000 DNA marker.

PCR identification for *SEq*

SEq is an enterotoxin gene of *S. aureus*. This enterotoxin was observed less frequently than were the other genes. The length of the PCR-amplified fragment of *SEq* was 222 base pairs,

as indicated in Figure 3. Twenty-one bacterial strains containing the enterotoxin *SEq* gene, with a detection rate of 9.7%, including 8 strains in fresh meat and meat products, 6 strains in quick-frozen food, 6 strains in raw milk, and 1 strain in human purulent tissue.

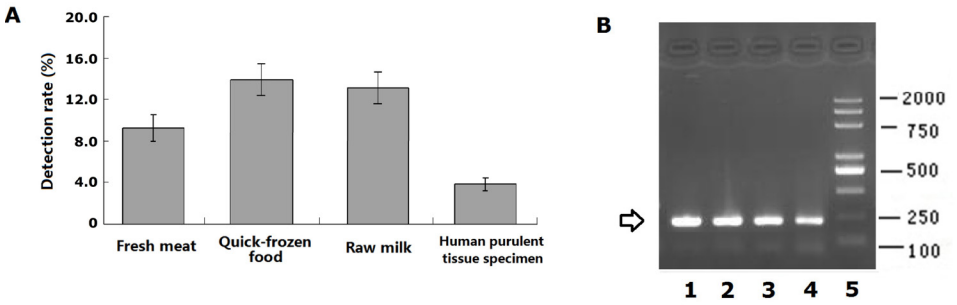

Figure 3. PCR amplification result of *SEq*. The detection rate in Quick-frozen was the highest. The length of the amplification fragment was 222 bp. *Lanes 1-3* = *SEq* gene. *Lane 4* = standard strain of *S. aureus*. *Lane 5* = DL2000 DNA marker.

The carrier rates of the 5 genes varied in the 217 strains of *S. aureus* from different origins. Some values were as low as zero. The carrier rates of 3 enterotoxins, *SEj*, *SEr*, and *SEq*, were the highest in quick-frozen food, followed by in fresh meat and meat products. No genes for enterotoxin were detected in bean products. *SEm* and *SEl* are uncommon genes in *S. aureus* enterotoxins. In the study, no bands for these genes were observed in the electrophoresis results. No *S. aureus* enterotoxins were detected after initial inspection, re-inspection, and final inspection. The detection rate of *SEj* was the highest in the 217 strains carrying target-gene enterotoxins. Ten strains contained 3 genes and 14 bacterial strains carrying *SEr* and *SEj*. The growing environment for *S. aureus* significantly affected the formation of enterotoxin types. *S. aureus* from different foods varied substantially in enterotoxins and types. The *S. aureus* that generated the 3 enterotoxins originated in raw milk, fresh meat, and quick-frozen food. These specimens were primarily and secondarily processed foods that were rich in protein. In addition, the natural environment from which these specimens were collected was contaminated. Although some specimens were preserved at a low temperature, food storage containers were in unhygienic condition and the surrounding had man-made pollution.

DISCUSSION

With improvements in molecular techniques, numerous enterotoxins have been discovered (Wu and Su, 2014). However, most studies have focused on the several traditional genes rather than newly discovered enterotoxins (Rosengren et al., 2013; Wu et al., 2013). The detection rates of the 3 new genes examined in this study were very low compared to those of the traditional enterotoxin genes and neither *SEm* nor *SEl* was detected. This may be because of small sample sizes or the single origin, among other reasons (Ding et al., 2013). Our results were consistent with those for the new enterotoxins in food in Taiwan (Becker et al., 1998) and trends observed for several new enterotoxins in food (Letertre et al., 2003b). Typing research has been conducted for the new enterotoxins but there have been few detailed studies predicting enterotoxins and their origins. There have been a few studies of the origins of new enterotoxins (Fischer et al., 2009;

Solano et al., 2013). The detection rates of the other 3 genes were the highest in quick-frozen food, except for the 2 undetected genes, followed by in fresh meat. By detailing the sources of the 2 types of food, we found that the 3 detectable enterotoxin genes were the highest in beef and mutton. The result should be supported by further studies examining the functions of the 3 enterotoxins. Based on a report by Scherrer et al. (1998), *SEj* is the main enterotoxin gene in sheep and cattle with mastitis. Thus, infection with *S. aureus* and *SEj* may be associated with mastitis (Zschöck et al., 2005). Based on previous studies examining several other traditional genes in our laboratory, the enterotoxin genotypes are found more frequently in meat products and quick-frozen foods that are closely associated with the living environment and food (Zhang et al., 1998; Ahmady and Sahar Kazemi, 2013). We found that the 5 new detectable enterotoxin genes were extensively present in protein of animal origin compared to that from other origins. This is related to the animal characteristics and interaction with the living environment, operation environment for food processing, and storage environment for finished products (Aydin et al., 2011; Ding et al., 2013).

Additionally, the detection rate of *SEj* was high for the new enterotoxins but much lower than that for the traditional enterotoxins. Fourteen bacterial strains carried both *SEr* and *SEj* based on repeated verification. *SEr* and *SEj* are operons in the enterotoxin gene cluster in *S. aureus* (Lei et al., 2010; Reis et al., 2012; Kaminska et al., 2014). Both have synergistic effects, and there is a synergistic effect between *SEj* and multiple enterotoxin genotypes. Thus, there is a high level of correlation and a synergistic relationship between *SEj* and *SEr*. However, the experimental data are limited and further studies should examine the relationship between *SEr* and *SEj*. We found that *SEj* had 6 sources, which also provides a basis for studying the correlation between *SEr* and *SEj*.

In this study, we examined the distribution characteristics of different food-borne *S. aureus* and 5 enterotoxin genes. The detection rate of *S. aureus* in food of animal origins was significantly higher than that in food of plant origins, but we found no significant difference among the detection rates of enterotoxin genes.

Only one bacterial strain could be separated from one specimen. Generally, different target bacterial strains separated from one specimen are the same strain. However, there are differences in the physicochemical properties and biological properties among different target bacterial strains separated from the same specimen. The difference is particularly significant regarding biological properties. For example, enterotoxins were identified for the *S. aureus* separated from the same specimen. Some tested negative, while some tested positive, and thus different types of *S. aureus* enterotoxins were separated from the same specimen. Therefore, the proper number of bacterial strains to be screened from the same specimen should be further examined.

The detection rates of the 5 genes detected were very low and even zero; however, this does not indicate that the 5 genes play no role in the toxicity effect and toxic reactions. The correlations among genes and among their origins should be compared in further studies.

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