

Prevalence of Enterotoxin Genes and Antibacterial Susceptibility Pattern of *Staphylococcus aureus* Strains Isolated from Animal Originated Foods in West of Iran

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ABSTRACT

Objectives: The aims of our study were to evaluate the prevalence of *Staphylococcus aureus* (*S. aureus*) strains in food samples of animal origin, examine their antibacterial susceptibility pattern, and to detect staphylococcal enterotoxin (SEs) genes and the *mecA* gene in isolated *S. aureus* strains using the polymerase chain reaction (PCR). **Methods:** A total of 1050 food samples including 671 raw milk and dairy products and 379 raw meats were collected between September 2013 and June 2014 in Hamadan, Iran. Food samples were analyzed for *S. aureus* identification. The antibiotic susceptibility pattern of all isolates was determined using the disk agar diffusion method followed by detecting *mecA* resistance gene using PCR. In addition, harboring of SE genes were determined using a multiplex PCR assay targeting nine genes. **Results:** A total of 98 (9.3%) *S. aureus* strains were isolated from 1050 food samples. Of the 98 isolates examined, the most frequent resistance was observed to erythromycin (30.6%), followed by tetracycline (29.6%), gentamicin (27.6%), clindamycin (26.5%), ciprofloxacin and rifampin (24.5%), trimethoprim-sulfamethoxazole (14.3%), and ceftiofloxacin (5.1%). All ceftiofloxacin resistant isolates were positive for *mecA*. The prevalence of SEs was 77.6% (n=76). Among the genes that code classic enterotoxins, *sea* was the most frequent and was carried by 25.5% of isolates, followed by *see* in 18.4%, *sed* in 11.2%, *sec* in 5.1%, and *seb* in 4.1% of isolates. Among the detected enterotoxins, *seg* was the predominantly identified enterotoxin gene in isolates with prevalence of 35.7%. The *seb* gene with prevalence of 1% and *sei* gene with 3.1% were other detected enterotoxins with low frequencies. **Conclusions:** The high prevalence of SE genes detected indicates a potential risk for causing animal-originated food poisoning. The increasing prevalence of community-acquired MRSA and its emerging antibiotic resistance in foods is a serious problem for public health.

The *Staphylococcus* genus belongs to the *Micrococcaceae* family. These bacteria are characteristically Gram-positive, non-motile, non-spore forming, and aerobic or facultatively anaerobic.¹ More than 20 species have been recognized in this genus, which are distributed in various habitats. Some colonize the skin surface, cutaneous glands, and human or animal (such as ruminants) mucosal membranes. In animals, such as birds and mammals, the skin and the mucous membranes (especially those of the nasopharyngeal region) are considered as the primary reservoirs for *Staphylococcus aureus* (*S. aureus*); thereby, this microorganism has enough potential to exist and transmit to or contaminate and spoil animal products such as milk, dairy products, and meats.

This is especially true of handled foods during the processing, preparing, wrapping, mincing, and storage steps, which require manipulation. Generally, due to the fact that *Staphylococcus* can grow in any condition without special or complicated requirements, a high variety of foodstuffs can be contaminated by this microorganism, including meat and meat products especially hamburgers, milk and dairy, chicken, fish, salted and fermented products, and even cooked foods (which account for 24% of all cases). In addition, this microorganism can transmit to environmental sources such as soil, sand, dust, and surface waters.²⁻⁴

S. aureus is capable of producing one or more of a family of enterotoxins that known as Staphylococcal enterotoxins (SEs). SEs are classified to classic

enterotoxins (SEA–SEE) and new enterotoxins (SEG–SEQ and SER–SEU) based on their biological and serological characteristics. From these twenty types of SEs, A to E are highly isolated from outbreaks of food poisoning in more than 90% of cases. According to studies performed in USA and UK, SEA and SEB have been isolated in 69% of food poisoning cases, while new enterotoxins were causes in 5% of cases.^{5,6} SEs are considered significant virulence factors belonging to a large family of pyrogenic toxin super antigens (PTSAgs).⁷

In order to detect SEs from isolated strains and food, several phenotypic methods have been introduced including immunological methods such as agglutination and biological and chromatographic assays. However, the major limitations of these methods are their low accuracy and lack of comprehensiveness. For example, in agglutination due to the fact that different SEs serotypes are partly similar antigenically, cross reactive reactions can occur. In addition, there are only five serologic kits (for SEA to SEE serotypes) available commercially. Therefore, using molecular assays such as the polymerase chain reaction (PCR) can be more accurate and useful than phenotypic methods for detecting enterotoxin producing genes in *S. aureus* strains.^{8,9}

Methicillin-resistant *S. aureus* (MRSA) is considered one of the most common pathogens causing nosocomial infection.^{10,11} MRSA strains are categorized into two families. The first family is known as the community-acquired MRSA (CA-MRSA) strains and are associated with strains acquired in community settings and are expected to be sensitive for many antibiotics. The second family are the hospital-acquired MRSA strains which originate from hospital infections and are resistant to many antibiotics. Foodstuffs have been considered as sources of MRSA strains, therefore, surveying and surveillance activities for foodstuffs harboring MRSA strains is needed.^{12–14}

We aim to evaluate the prevalence of *S. aureus* strains in food samples of animal origin and to examine their antibacterial susceptibility pattern as well as to use PCR for the molecular detection of the SEs genes and the *mecA* gene in the isolated strains.

METHODS

A total of 1050 food samples were purchased and collected between September 2013 and June 2014,

in Hamadan, Iran. The samples were kept in an insulated ice box and transferred to the laboratory and examined within 1–2 hours for the presence of *S. aureus* according to the Iran national standard (number=1194) for *S. aureus* identification.

Processing of collected samples for isolation of *S. aureus* was carried out as follows: approximately 10g of each meat samples were added to test tubes containing 90ml sterile buffered peptone water (BPW; Merck Co., Darmstadt, Germany) and homogenized using vortex mixer (other samples used specific protocols). Then 1ml of each yielded suspension inoculated in test tube containing enrichment media of Giolitti Cantoni broth (QUELAB Co., UK) supplemented with 3.5% potassium tellurite solution and incubated aerobically at 37°C for 24 and 48 hours. The test tubes presenting blackening throughout or in the bottom of the tube were subcultured to Baird-Parker Agar medium (BPA; Merck Co., Darmstadt, Germany) supplemented with 5% egg yolk tellurite emulsion (Merck Co., Darmstadt, Germany) and incubated aerobically at 37°C for 48 hours. Colonies with typical black appearance surrounded by a clearing zone were considered as *Staphylococcus* genus and were subcultured to blood agar (Merck, Co., Darmstadt, Germany). Colonies that grew in blood agar were identified as *S. aureus* with some phenotypic criteria. Finally, all presumed identified staphylococcal strains were tested for coagulating citrated rabbit plasma. *S. aureus* ATCC 25423 was used as positive control and *Staphylococcus epidermidis* as a negative control.⁴

Total DNA was extracted from overnight-grown pure isolates using the DNA extraction Kit (BioFlux Co., Tokyo, Japan), according to the manufacturer's instructions. The quality of extracted DNA was assessed spectrophotometrically by the Nanodrop ND-1000 (Nanodrop Technologies, Inc., Delaware, USA) using 2% agarose gel in 1x TBE buffer.

Following the DNA extraction, to confirm all presumed identified isolates as *S. aureus* species, a PCR assay targeting the *S. aureus*-specific *nuc* gene coding deoxyribonuclease enzyme¹ was performed. The reaction mixture for PCR assay was 20µL and was prepared as follows: 10µl of 2x Taq premix Mastermix (Parstous Biotech Co., Iran), 5µl of sterile double-distilled water, 1µl of forward primer, 1µl of reverse primer, and 3µl of DNA sample. The DNA samples as well as a positive control (*S. aureus* ATCC

25423) and a negative control (*S. epidermidis*) were amplified by an initial denaturation step for 5 minutes at 94°C followed by 35 cycles of 94°C for 60 seconds, 50°C for 60 seconds, 72°C for 1 minute, and a final extension step at 72°C for 10 minutes in a Bio-Rad Thermal Cycler (Bio-Rad Laboratories, Inc., USA).¹⁵ After the amplification reaction, 5µl of each PCR product were visualized and photographed with an UV transilluminator (VilbertLourmat Co., Japan)

For all confirmed *S. aureus* isolates, antibacterial susceptibility testing were carried out by standard Kirby-Bauer disk agar diffusion (DAD) on Mueller Hinton agar (Merck Co., Germany) according to the Clinical and Laboratory Standards Institute (CLSI, 2013)¹⁴ for the antibiotics including cefoxitin (30µg), gentamicin (30µg), erythromycin (15µg), ciprofloxacin (5µg), rifampin (5µg), trimethoprim-sulfamethoxazole, trimethoprim-sulfamethoxazole (SXT) (1.25/23.75µg), clindamycin (2µg), and tetracycline (30µg). All antibiotics were from MAST Co., England. Methicillin-resistance was examined using cefoxitin DAD and confirmed by *mecA*-specific PCR assay.¹⁴

The PCR assay targeting *mecA* gene coding methicillin-resistance was performed for all isolates.^{15,16} The reaction mixture for the single target amplification PCR assay was performed as previously described.

Three multiplex PCR assays targeting a total of nine SEs genes were designed: multiplex I containing *sea*, *sei*, and *seg*; multiplex II containing *see*, *sec*, and *seb*; and multiplex III containing *seb*, *sed*, and *sej* primer pairs. All primer sequences have been listed in Table 1. Each multiplex PCR reaction carried out in a volume of 25µl that contained: 12.5µl of 2x Taq premix Mastermix, 3.5µl sterile double distilled water, 1µl of each forward primers, 1µl of each reverse primers, and 3µl of DNA sample. In each PCR reaction, the DNA samples as well as a negative control were amplified by an initial denaturation of DNA at 95 °C for 5 minutes and was followed by 35 cycles of amplification (95°C for 30s, 55°C for 30s and 72°C for 60s), and a final extension at 72°C for 10 minutes.¹⁷ The amplified PCR products were resolved using electrophoresis and visualized as previously above described.¹⁷

Table 1: Primer sequences used for the polymerase chain reaction (PCR) assay in this study.

Target gene	Sequences	Gene accession no.	Product (bp)	Annealing temperature (°C)	Reference
<i>nuc</i>	F: GCGATTGATGGTGATACGGTT R: AGCCAAGCCTTGACGAACTAAAGC	DQ399678	279	50	Hennekinne et al, ¹
<i>mecA</i>	F: GTAGAAATGACTGAACGTCCGATAA R: CCAATTCCACATTGTTTCGGTCTAA	X52593	310	55	Hennekinne et al, ¹
Multiplex I					
<i>sea</i>	F: GAAAAAAGTCTGAATTGCAGGGAACA R: CAAATAAATCGTAATTAACCGAAGGTTC	M18970	560	55	Argudín MA et al, ³
<i>sei</i>	F: GTACCGTTGAAAATTCAG R: AGGCAGTCCATCTCCTG	AAW3643/ AF285760	461	55	Bianchi et al, ⁴
<i>seg</i>	F:TCTCCACCTGTTGAAGG R: AAGTGATTGTCTATTGTCTG	AF285760	323	55	Bianchi et al, ⁴
Multiplex II					
<i>see</i>	F: CAAAGAAATGCTTTAAGCAATCTTAGGC R: CACCTTACCGCCAAAGCTG	M21319	482	55	Argudín et al, ³
<i>sec</i>	F:CTTGATGTATGGAGGAATAACAAAACATG R: CATATCATAACAAAAGTATTGCCGT	X05815	275	55	Argudín et al, ³
<i>seb</i>	F: CAATCACATCATATGCGAAAGCAG R: CATCTACCCAAACATTAGCACC	U11702	376	55	Argudín et al, ³
Multiplex III					
<i>sed</i>	F:GAATTAAGTAGTACCGCGCTAAATAATATG R: GCTGTATTTTTCTCCGAGAGT	M28521	492	55	Argudín et al, ³
<i>seb</i>	F:ATTCTATTAAGGACACTAAGTTAGGGA R: ATCCCGTTTCATAAGGCGAGT	M11118	404	55	Argudín et al, ³
<i>sej</i>	F: TCAGAAGTGTGTTCCGCTAG R: GAATTTTACCAYCAAAGGTAC	AF053140	138	55	Bianchi et al, ⁴

Table 2: Information of the collected samples and their polymerase chain reaction (PCR) assay results.

Food types	Samples, n	Isolates n (%)	<i>mecA</i> isolates, n	Frequency of enterotoxin type detected by multiplex PCR assay (n)												Total samples with <i>se</i> (%)	
				<i>sea</i>	<i>seb</i>	<i>sec</i>	<i>sed</i>	<i>see</i>	<i>seg</i>	<i>seb</i>	<i>sei</i>	<i>sej</i>	<i>sec</i> + <i>sed</i>	<i>sea</i> + <i>seg</i>	<i>sea</i> + <i>see</i> + <i>seg</i>		<i>sea</i> + <i>see</i> + <i>seg</i> + <i>sej</i>
Cream	66	4 (6.1)	0	1	0	0	1	0	0	1	0	0	0	0	0	0	4 (100.0)
Traditional yoghurt	45	2 (4.4)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1 (50.0)
Raw milk	271	29 (10.7)	3	5	1	2	2	1	4	0	2	0	2	0	3	0	22 (75.9)
Raw red meat	243	25 (10.3)	0	3	0	1	2	3	6	0	1	0	1	1	1	1	19 (76.0)
Raw poultry	136	11 (8.1)	0	2	0	0	1	1	3	0	0	0	0	0	1	0	8 (72.7)
Traditional cream	47	2 (4.3)	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1 (50.0)
Cheese	170	19 (11.2)	2	0	1	2	3	3	3	1	1	1	1	0	3	0	16 (84.2)
Butter	72	6 (8.3)	0	0	2	0	0	0	2	0	0	0	1	0	0	0	5 (83.3)
Total (%)	1050	98 (9.3)	5 (5.1)	25 (25.5)	4 (4.1)	5 (5.1)	11 (11.2)	18 (18.4)	35 (35.7)	1 (1.0)	3 (3.1)	0	1 (1.0)	5 (5.1)	8 (8.16)	1 (1.0)	76 (77.6)

For confirmation of PCR product, one sample of each of the enterotoxins gene and *mecA* PCR products (amplicons) were sequenced (Bioneer Co., Korea) and the data were analyzed using the Chromas software.

All categorical (continuous) variables were compared using the 2-tailed chi-square test or Fisher's exact test; *p*-values of ≤ 0.050 were considered statistically significant. All statistical analyses were performed using the SPSS (SPSS Inc., Chicago, USA) version 20 software package.

RESULTS

A total of 98 (9.3%) *S. aureus* strains from 1050 food samples were isolated, identified and confirmed by PCR assay targeting the *nuc* gene [Figure 1]. They included 36 (9.5%) isolates from the 379 raw meat samples and 62 (9.2%) isolates from the 671 raw milk and dairy product samples. The total number of *S. aureus* isolated among the various collected food type samples has been shown in Table 2.

Table 3 shows the antimicrobial susceptibility patterns of the 98 *S. aureus* isolates examined. The *mecA* gene was detected in all MRSA strains, which composed 5.1% of all *S. aureus* strains [Figure 1].

Prevalence of SEs was determined to be 77.6% (n=76); 79.0% (n=49) in raw milk and dairy products and 75.0% (n=27) in raw meat samples. The percentages of enterotoxigenic strains isolated can be found in Table 2.

The genes of classical enterotoxins (*sea*, *seb*, *sec*, *sed*, and *see*) were carried in 63 (64.3%) isolates and

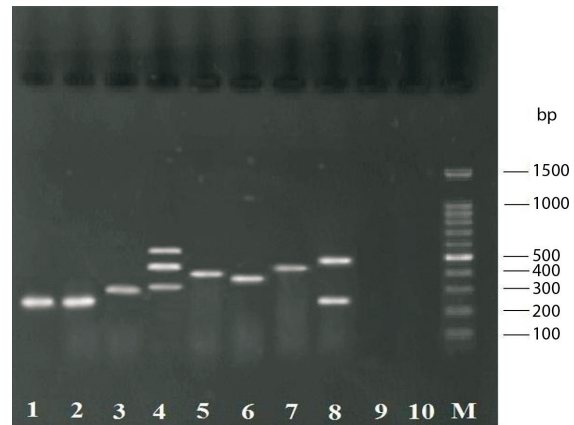


Figure 1: Gel electrophoresis for PCR assays products. Lane M: 100-bp DNA size marker; lane 1: 279-bp amplicon of *nuc* for *S. aureus* positive control (ATCC 25423); lane 2: 279-bp amplicon of *nuc* for food sample; lane 3: 310-bp amplicon for *mecA*; lane 4: 323-, 482- and 560-bp amplicons for *seg*, *see* and *sea*, respectively; lane 5: 404-bp amplicon for *seb*; lane 6: 376-bp amplicon for *seb*; lane 7: 461-bp amplicon for *sei*; lane 8: 275- and 492-bp amplicons for *sec* and *sed*, respectively; line 9: *S. epidermidis* (negative control); and lane 10: sample without DNA template (negative control).

those of new enterotoxins (*seg*, *seh*, *sei*, and *sej*) were carried in 39 (39.8%) of isolates [Figure 1]. Among the genes that code classic enterotoxins, *sea* gene was the most frequent and was carried by 25 (25.5%) isolates [Table 2].

All our sequenced strains with related PCR product showed the same DNA sequences therefore all PCR assay results were confirmed.

DISCUSSION

A wide variety of enterotoxin and enterotoxin-like coding genes (more than 20 SEs) have been recognized to have a significant role in stages of host colonizing, gastroenteritis infections, and invasion of skin, mucus, and host defence mechanisms.^{3,17,18}

Food is regarded a significant factor for transferring and spreading of antibiotic resistance genes. *S. aureus* carrying virulence and antibiotic resistance genes on mobile genetic elements such as plasmids, prophages, and staphylococcal pathogenic islands (SaPIs), and these can horizontally transfer between strains. Consequently, investigation of the antibiotic resistance patterns of *S. aureus* strains is essential in planning MRSA control measures.^{19,20}

Table 3: Results of antimicrobial susceptibility of *Staphylococcus aureus* using disk diffusion method.

Antibiotics	Number (%) of <i>S. aureus</i> isolates (n=98)		
	Resistant	Intermediate	Sensitive
Cefoxitin	5 (5.1)	nd	93 (94.9)
Erythromycin	30 (30.6)	3 (3.1)	65 (66.3)
Tetracycline	29 (29.6)	1 (1.0)	68 (69.4)
Gentamicin	27 (27.6)	nd	71 (72.4)
Clindamycin	26 (26.5)	1 (1.0)	71 (72.4)
Ciprofloxacin	24 (24.5)	nd	74 (75.5)
Rifampin	24 (24.5)	4 (4.1)	70 (71.4)
SXT	14 (14.3)	2 (2.0)	82 (83.7)

nd: not detected; SXT: trimethoprim-sulfamethoxazole

In our study, we aimed to determine the prevalence of enterotoxin producing *S. aureus* strains collected from foods of animal origin and characterize them. Several phenotypic and genotypic methods were used to detect the presence of enterotoxin and antibiotic resistance genes and to determine the antibacterial susceptibility pattern.

The 9.3% prevalence rate of *S. aureus* in our study was similar to a study performed in Tehran, which reported a prevalence of 9.5%.²¹ A study performed in Turkey reported the prevalence of *S. aureus* in food samples was 13.8%.²² In another study in Canada between 2007 and 2010, the prevalence of *S. aureus* was 10.5%.²³

The contamination rate of raw meat and dairy product samples (9.5% and 9.2%, respectively) was not statistically significant ($p > 0.050$). However, a study by Dallal et al,²¹ reported a significant difference with a prevalence of 17.1% for dairy products and 3.5% for meat and other products. In a study performed in Italy by Bianchi et al,⁴ the prevalence of *S. aureus* was reported as 39% in milk and dairy products, which was higher than our study. A study in Turkey reported a prevalence of 57.3%.²⁴

This inconsistency in the reported prevalence of *S. aureus* between meat and dairy product samples can be explained by contaminated milk and dairy products via cows and/or sheep with mastitis and during the pasteurization step by human/environmental sources or instruments and equipment with a low standard of cleanliness. Furthermore, cheeses produced from raw and unpasteurized milk are considered significant agents in the spread of *S. aureus* food poisoning.

Of the 98 *S. aureus* strains examined in this study, five (5.1%) harbored the *mecA* gene. Each *mecA* positive strain demonstrated resistance to cefoxitin (i.e. CA-MRSA). All CA-MRSA strains were isolated from dairy product samples rather than raw meat samples ($p < 0.050$). The following rates of CA-MRSA were reported in other similar studies; 6.4% in Canada, 3.8% in Italy, 2.5% in the Netherlands, 1.6% in Spain, and 2.4% in Korea.²³

According to several studies, the main factor in selecting for antibiotic-resistant bacteria is prescribing antibiotics in animal husbandry for therapeutic purposes or as growth promoters. Multiple antimicrobial resistance patterns have been found in *S. aureus*.^{14,23}

There was no statistically significant difference

between raw meat and dairy product samples in antibacterial susceptibility patterns. In addition, statistical analysis comparing antibiotic resistance patterns between CA-MRSA and methicillin-sensitive *S. aureus* (MSSA) isolates revealed that the CA-MRSA strains were significantly more resistant to antibiotics erythromycin, tetracycline, gentamicin and rifampin than the MSSA strains. This finding was in agreement with a study performed by Dejing et al,²⁵ in 2010. Although resistance to antibiotics ciprofloxacin and SXT were higher in the CA-MRSA strains than that of MSSA strains these differences were not statistically significant. Clindamycin was the only exception as the resistance rate in MSSA strains were in some cases higher than that of CA-MRSA strains.

We assessed gene coding for enterotoxins A–E and G–J amongst the isolated *S. aureus*. Strains revealed a wide variability in carrying *se* genes; 77.6% of the strains were carrying one or more of the toxin genes tested. This result was partly consistent with finding in another study that reported 84.9% of foodborne strains harbored enterotoxin.²⁶ In a Portuguese study, the prevalence of enterotoxigenic strains was reported 68.2%⁹ and another study performed in Italy reported a 59.8% prevalence of *se* genes in food samples.²⁷ In Japan, Katsuda et al,²⁸ found 183 (67.8%) out of 270 enterotoxigenic *S. aureus* isolates.

Based on studies, *se* genes can be located on plasmids (*sed* and *sej*), phages (*sea* and *see*), pathogenicity islands (*seb* and *sec*) and chromosomes (*seg*, *seh*, and *sei*); thereby, several *se* genes can be harbored by enterotoxigenic *S. aureus* strains.¹⁹

In our study, SEA was the most frequently observed classic SE in enterotoxigenic *S. aureus* strains. In addition, *seg* gene was predominant enterotoxin in case of newly identified enterotoxin genes and it was also the most prevalent enterotoxin in our study generally.

SEA, a major leading cause of food poisoning,^{29,30} is carried by prophage Sa3mu alone or by Sa3mw with other genes (*seg* and *sek*).³¹ In our study, the *sea* gene was detected in 25.5% (n=25) of *S. aureus* strains, and also in coexistence with *seg* gene in 14.3% (n=14). Enterotoxin SEH encoded on the chromosome.¹⁹ In our study, it was detected only in one strain isolated from cheese. This finding was consistent with the study performed by Alibayov et al.¹⁹ The *seg* and *sei* genes are usually detected

together¹⁹; however, in this study these two genes were detected separately.

In current work, the rate of detected classical enterotoxin genes was higher than that of new enterotoxins (64.3% and 39.8%, respectively). In addition, seven (7.1%), eight (8.2%) and one (1.0%) isolates were simultaneously positive for two, three, and four *se* genes, respectively. These results are in accordance with findings of the study by Wu et al.²⁵

The detection of SE genes indicates diversity of the prevalence of *se* genes in *S. aureus* strains isolated from animal food samples in Hamadan. In fact, there were differences in the patterns of *se* genes in relation to the origin of the isolates. It is known that the patterns of SE genes are variable between different geographical origins and years. This difference can be explained by host adaptations of *S. aureus* in different animal species.^{13,27,32}

There was a significant correlation between *S. aureus* isolates from butter samples and harboring *se* genes ($p < 0.035$). However, no significant differences were observed between *S. aureus* isolates from other food samples and harboring *se* genes. Additionally, there was no statistically significant difference between raw meat and dairy product samples for harboring *se* genes.

A statistically significant correlation was found between some antibiotic susceptibility patterns and carrying *se* genes in examined isolates. This included between cefoxitin and carrying *sea* and *see* genes ($p = 0.001$) and between erythromycin and carrying *sea*, *sed*, and *see* genes, tetracycline and carrying *sea* and *see* genes, gentamicin and carrying *sea* gene, rifampin and carrying *sec* and *see* genes, and between SXT and carrying *sea* gene ($p < 0.050$).

CONCLUSION

S. aureus accounts for frequent contamination of food products and is a well-known clinical and epidemiological pathogen. The detection of the high rate prevalence of enterotoxin genes in this study indicates a potential risk for causing animal originated food poisoning. The increasingly prevalence of CA-MRSA and its emerging antibiotic resistance in foods is a serious problem for public health. Infected animals and infection during the processing stage are the most frequent transmission routes in food contamination with *S. aureus* or MRSA. Therefore, continuous surveillance and

monitoring of pathogens with potential to infect foods and the emergence of MRSA and antibiotic resistance is necessary for public health. For this purpose, the utilization of antibiotics as growth promoters in animal husbandry should be avoided especially those frequently used for caring for both humans and animals.

Disclosure

The authors declared no conflicts of interest.

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