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Frequency of Enterotoxin Producing *Staphylococcus aureus* and Toxin Genes in Raw and Cooked Meat Samples

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ABSTRACT

Aims Food safety has emerged as an important global issue with international trade and public health implications. *Staphylococcus aureus* is recognized as an important cause of food poisoning related to the consumption of raw, undercooked or mishandled foods worldwide. The aim of this study was to investigate the presence and the frequency of enterotoxin producing *S. aureus* and SE genes in meat samples collected from meat retail outlets and restaurants in Zanjan, Iran.
Materials & Methods In this cross sectional study, from March to June 2015, a total of 90 individual meat samples were collected from meat retail outlets and restaurants in Zanjan, Iran and investigated the frequency of enterotoxin producing *S. aureus* and SE genes. The meat samples were immediately homogenized and cultured on Baird parker agar and subjected for confirmatory biochemical tests and molecular detection of *femA*, *sea*, *seb*, *sec*, *sed* and *see* genes.
Findings A total of 31 (34.4%) meat samples were positive for the presence of *S. aureus*. The frequency of *S. aureus* in raw meat (23.3%) was higher than cooked meat samples (11.1%). Enterotoxin-producing capacity was determined in 18 (20.0%) out of 90 homogenized meat samples using ELISA technique. The most prevalent SE gene was *sea* (38.7%), followed by *see* (22.6%), *sec* (16.1%) and *seb* (12.9%). SE genes were not found in strains isolated from cooked meat samples.
Conclusion Detection of enterotoxigenic *S. aureus* in raw meat samples shows a probable risk for public health.

Keywords *Staphylococcus aureus*; Enterotoxins; Meat; PCR

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Introduction

Staphylococcus aureus is one of the most common causes of bacterial food poisoning outbreak [1, 2]. Staphylococcal food poisoning (SFP) is related to the consumption of foods containing sufficient amounts of one or more enterotoxins [3-6]. The high incidence of staphylococcal food poisoning is due to the insufficient pasteurization/decontamination of originally contaminated product source or its contamination during preparation and handling by individuals who are carriers of the organism [7].

Foods that have been frequently incriminated in staphylococcal intoxication include meat and meat products, poultry and egg products, milk and dairy products, salads, bakery products, particularly cream-filled pastries and cakes, and sandwich fillings [8]. *S. aureus* produces a wide variety of toxins belong to the fascinating family of superantigens including staphylococcal enterotoxins (SEs; SEA-SEE, SEG-SEI and SER-SET) and staphylococcal enterotoxin like (SEls) toxins, which their emetic properties have remained unconfirmed (SElK-SElQ and SElU-SElX). SEs and SEls are single chain proteins with size range from 22-29KDa and encoded by accessory genetic elements including plasmids, prophages, pathogenicity islands, genomic island vSa, or by genes located next to the staphylococcal cassette chromosome (SCC) [8-11].

The amount of SE required for establishment of typical symptoms of food poisoning including nausea, vomiting, emesis, stomach cramps and diarrhea is very low and approximately ranging from 20ng to 1µg [3, 12, 13]. Among SEs, SEA is the most common cause of staphylococcal food poisoning worldwide, but the involvement of other classical SEs has been also demonstrated [8]. Due to the stability of SEs in denaturing conditions such as heat and low pH, these toxins are not completely destroyed by mild cooking or digestion of food in the stomach [14, 15].

Although enterotoxigenic staphylococci are thermally destroyed, the cooked meat products may contain SEs because these toxins are thermostable and cannot be destroyed by heat processing. This fact represents a serious hazard to healthy consumer when ready-to-eat meat products are processed [16]. Therefore, it is essential to detect SEs-producing staphylococci and gather information about other microbial risk factors and hazards associated with raw and pre-processed meat products. Risk assessment and microbial monitoring will continue to play important role in quality assurance of meat products [16].

The detection of *S. aureus* and SEs in food is difficult. Methods currently used for detection of SEs in food are Enzyme-Linked Immunosorbent Assay (ELISA), reversed passive latex agglutination (SET-RPLA) and polymerase chain reaction (PCR) technique [14].

In this study, we determined the prevalence of *S. aureus* and frequency of SE-genes (*sea*, *seb*, *sec*, *sed* and *see*), in food isolates (beef, lamb and cooked meat) from Iranian markets, restaurants and other food distribution centers to determine the ability of the isolates to produce classical staphylococcal enterotoxins SEA–SEE by PCR method. In addition, a commercially available kit RIDASCREEN® SET total (SEA-SEE) was employed for the detection of Total staphylococcal enterotoxins A to E by sandwich enzyme immunoassay (ELISA) technique.

The aim of this study was to investigate the presence and the frequency of enterotoxin producing *S. aureus* and SE genes in meat samples collected from meat retail outlets and restaurants in Zanjan, Iran.

Materials and Methods

In this cross sectional study, from March to June 2015, a total of 90 individual meat samples including 23 raw beef, 22 raw lamb and 45 cocked meat samples were collected from meat retail outlets and restaurants in Zanjan, Iran. Meat samples were packed into a clean polyethylene bag then marked and transported to the laboratory of food microbiology in a cool box for analysis within 1h.

Reference strains: Reference strains of *S. aureus* ATCC 13565 (SEA), *S. aureus* ATCC 14458 (SEB), *S. aureus* ATCC 19095 (SEC), *S. aureus* ATCC 23235 (SED) and *S. aureus* ATCC 27664 (SEE) were used as positive controls in the study.

Isolation and identification of *S. aureus*: Twenty five gram of meat samples was homogenized for 90s in a stomacher (Heidolph; Schwabach; Germany) with 225mL of peptone water (PW) containing 6.5% NaCl and then incubated at 37°C for 24h. After primary enrichment, a loopful (without shaking the flask) from each of the enriched homogenates was streaked onto Baird-Parker agar (MERCK; Darmstadt; Germany) supplemented with 5% egg yolk and tellurite and incubated under aerobic conditions at 37°C for 24h. Colonies with typical grey-black appearance surrounded by a clear zone were enumerated as coagulase positive staphylococci and sub-cultured onto Mannitol salt agar (Merk; Darmstadt; Germany). The isolates were identified as *S. aureus* by further biochemical characterization using Gram stain, catalase, coagulase, oxidase, lipase, DNase and PCR targeting the *S. aureus* specific *femA* gene (*S. aureus* species specific).

Genomic DNA extraction: A colony of S. aureus (one colony per sample) was picked from nutrient agar and inoculated into 5ml of LB (Luria Bertani Broth; Merck; Germany) and incubated with

shaking at 120rpm at 37°C. Extraction of genomic DNA was performed according to the protocol provided with the Qiagen Mini Amp kit (Qiagen Inc.; Germany).

Detection of *sea-see* in *S. aureus* isolates by PCR: The presence of staphylococcal enterotoxin genes; *sea*, *seb*, *sec*, *sed* and *see* was assessed using the primers (Table 1) [17, 18].

Table 1) Primers used in the study

Target	Primer sequence (5'→3')	Amplicon size (bp)
femA	AAAAAAGCACATAACAAGCGGATAAAGA AGAAACCAGCAG	132
sea	CCTTTGGAAACGGTTAAAACGTCTGAACC TTCCCATCAAAAAC	127
seb	TCGCATCAAACTGACAAACGGCAGGTACT CTATAAGTGCC	477
sec	CTCAAGAACTAGACATAAAAGCTAGG TCAAAATCGGATTAACATTATCC	271
sed	CTAGTTTGGTAATATCTCCTTTAAACGTT AATGCTATATCTTATAGGGTAAACATC	319
see	CAGTACCTATAGATAAAGTTAAAACAAG CTAACTTACCGTGGACCCTTC	178

Single PCR was performed using DreamTaq PCR Master Mix (Thermo Fisher Scientific), which contains Taq polymerase, dNTPs, $MgCl_2$ and the appropriate buffer. Each PCR tube contained 25µl reaction mixture composed of 12.5µl of the master mix, 2.5µl of each forward and reverse primer solution (in a final concentration of 200nM), 2µl of DNA with concentration of 400ng and nuclease-free water to complete the final volume. PCR was performed using the Gene Atlas 322 system (ASTEC) with the same cycling conditions for *sea-see* genes.

Amplification involved an initial denaturation at 94°C, 5min followed by 30 cycles of denaturation (94°C, 1.5min), annealing (55°C, 1.5min) and extension (72°C, 1.5min), with a final extension step (72°C, 8min). The amplified DNA was separated by submarine gel electrophoresis on 1.5% agarose, stained with ethidium bromide and visualized under UV transillumination.

Detection of SEA-SEE enterotoxins: Staphylococcal enterotoxins (SEA, SEB, SEC, SED and SEE) were determined in homogenized meat samples by using ELISA technique (Thermo; Finland) with commercially available kit (Ridascreen® SET total; R-Biopharm AG; Darmstadt; Germany, Art. No. R4105).

Findings

Frequency of *S. aureus* in meat samples: A total of 90 individual meat samples were studied for the presence of *S. aureus*. Conventional cultural method based on appearance of grey-black colonies surrounded by a clear zone on Baird Parker agar plates were detected coagulase positive staphylococci in 43 (47.8%) out of the 90 samples. However, the biochemical tests and molecular analysis of *femA* in coagulase positive staphylococci indicated that 34.4% (31/90) of samples were positive for *S. aureus*: 12 (13.3%) isolates from raw lamb, 9 (10.0%) isolates from raw beef and 10 (11.1%) isolates from cooked meat samples (Table 2).

Frequency of total enterotoxins (SEA-SEE) in meat samples by ELISA technique: Of 18 enterotoxin positive samples, 10 (11.1%) were homogenized lamb and 8 (8.9%) beef samples. Total classical enterotoxins were not found in cooked meat samples (Table 2).

Distribution of enterotoxin genes (*sea*, *seb*, *sec*, *sed* and *see*) in *S. aureus* isolates: Overall, 58.1% (18/31) of isolates were positive for the presence of at least one or more SEs genes: 10 isolates (32.3%) from lamb and 8 isolates (25.8%) from beef samples. Comparision of SE genes frequency among beef and lamb isolates showed different distribution of these genes. The most prevalent SE gene among beef and lamb isolates was *sea* (38.7%), followed by *see* (22.6%), *sec* (16.1%) and *seb* (12.9%). SE genes were not found in strains isolated from cooked meat samples (Table 3).

The presence of multiple SE genes with different combinations was found among isolates. Of 18 *S. aureus* isolates carrying enterotoxin genes, 10 (55.5%) isolates had two or more SE genes simultaneously. The frequent combination of SE genes was *sea+see* (16.7%), followed by *sea+seb+see* (11.1%). Furthermore, one isolate (5.5%) of lamb samples carried *sea*, *seb*, *sec* and *see* simultaneously (Table 4).

Table 2) Frequency of *S. aureus* and total enterotoxins (SEA-SEE) in meat samples

Meat type	No.	Samples containing *S. aureus*		Samples containing total enterotoxins	
		Positive samples	No. (%)	Positive samples	No. (%)
Raw lamb	22	A2, A3, A5, A7, A8, A9, A10, A14, A16, A18, A19, A21	12 (13.3)	A2, A5,A7, A8, A9, A14, A16, A18, A19, A21	10 (11.1)
Raw beef	23	B1, B2, B3, B6, B8, B9, B10, B12, B13	9 (10.0)	B1, B2, B3, B6, B8, B9, B12, B13	8 (8.9)
Cooked meat	45	C5, C13, C16, C24, C26, C32, C34, C35, C39, C45	10 (11.1)	-	0
Total	90	A2, A3, A5, A7, A8, A9, A10, A14, A16, A18, A19, A21, B1, B2, B3, B6, B8, B9, B10, B12, B13, C5, C13, C16, C24, C26, C32, C34, C35, C39, C45	31 (34.4)	-	18 (20.0)

Table 3) Distribution of SE genes among 31 *S. aureus* isolates

Genes	Lamb isolates (n= 12)	Beef isolates (n= 9)	Cooked meat isolates (n= 10)	Total (n= 31)
sea	5 (16.1%)	7 (22.6%)	0	12 (38.7%)
seb	3 (9.7%)	1 (3.2%)	0	4 (12.9%)
sec	4 (12.9%)	1 (3.2%)	0	5 (16.1%)
sed	1 (3.2%)	1 (3.2%)	0	2 (6.4%)
see	3 (9.7%)	4 (12.9%)	0	7 (22.6%)

Table 4) Specific SE combinations among the 10 *S. aureus* isolates (5 beef and 5 lamb isolates) carrying more than one SE genes

SE combinations	Lamb isolates carrying SE genes (n=10)	Beef isolates carrying SE genes (n= 8)	Total (n=18)
sea+sed	-	1 (5.5%)	1 (5.5%)
sea+seb	1 (5.5%)	-	1 (5.5%)
sea+see	-	3 (16.7%)	3 (16.7%)
sec+sed	1 (5.5%)	-	1 (5.5%)
sea+seb+see	1 (5.5%)	1 (5.5%)	2 (11.1%)
sea+sec+see	1 (5.5%)	-	1 (5.5%)
sea+seb+sec+see	1 (5.5%)	-	1 (5.5%)

Discussion

Food safety has emerged as an important global issue with international trade and public health implications. *S. aureus* is a most common foodborne pathogen and represents a major public health problem in developing countries [1]. It is reported that raw meat and meat products are associated with staphylococcal food poisoning worldwide [8, 14].

In this study, a total of 31 (34.4%) meat samples were positive for the presence of *S. aureus*. The frequency of *S. aureus* in raw meat samples (23.3%) was higher than cooked meat samples (11.1%). Only a few reports on the frequency of *S. aureus* in meat samples from Iran have been previously published. According to the previous reports from Iran, 3.7-15.6% of the meat samples [19] were positive for the presence of this pathogen. Raw meat contamination with *S. aureus* has been reported 24% in Italy yielding positive cultures [20]. According to Moon *et al.* [21] and Aydin *et al.* [22], the frequency of *S. aureus* in meat products was 36% and 13.8%, respectively. This variation in *S. aureus* frequency may be due to differences in the geographical region, reservoir in the various countries, number of samples, seasons of sampling, post-harvest practices and hygienic standards applied during the handling, transport and storage of products, as well as the methods used for isolation and identification of this bacterium. Meat contamination may occur at various stages in preparation including transport, butchering and cut-up in the kitchen and the importance of chopping boards as a source of contamination has been reported [23].

In the present study, enterotoxin-producing capacity was determined in 18 (20.0%) out of 90 homogenized meat samples using ELISA technique. In contrast to our results, high frequency of enterotoxin producing *S. aureus* in food samples was detected in previous studies [24, 25]. According to Aydin *et al.* [22], 62.6% of *S. aureus* strains isolated from various food samples were enterotoxigenic. Similar results were reported by Guvent *et al.* [26] and Normanno *et al.* [27] which found the frequency of 60.1% and 59.8% of enterotoxigenic isolates from milk, dairy and meat products, respectively.

SEs genes were identified in 18 (58.1%) of 31 meat isolates. We found *sea* (38.7%) and *see* (22.6%) genes with higher frequency than others. Similar to our results, SEA is considered to be most common cause of food poisoning in Korea and Japan [28]. Furthermore, several studies reported that enterotoxin genes; *sea* and *sed* were the most common in staphylococci isolated from food [15, 24, 29]. However, lower incidence of *sed* (6.4%) in meat isolates was detected in our study. In contrast to our results, *seb* was a prevalent gene in food poisoning cases reported in Taiwan and Japan and *sec* was a major SE gene in isolates from bulk milk in Switzerland and in Korea [28]. In our study, SE genes were not found in strains isolated from cooked meat samples.

It has been known that the *se/sel* genes are carried on mobile genetic elements and most of them contain several *se/sel* genes simultaneously. SE genes are located on plasmids (*sed* and *sej*), phages (*sea*, *see* and *sep*) and pathogenicity islands on chromosomes (*seb*, *sec*, *seg*, *seh*, *sei*, *sek*, *sel*, *sem*, *sen*, *seo* and *seq*) [22, 30]. In our study, 55.5% of enterotoxin carrying isolates possessed more than one SE gene. Seven SE genotypes were observed, the most commonly detected were *sea+see* (16.7%) and *sea+seb+see* (11.1%).

According to our results and because of high prevalence of *S. aureus* (34.4%) and their entrotoxin genes in meat samples, intensive and continuous monitoring of pathogenic *S. aureus* is strongly recommended in order to evaluate the human health risk arising from food consumption. The limitation of our study was unavailability of ELISA kit and the number of samples.

Conclusion

Detection of enterotoxin producing *S. aureus* in raw meat marketed in Zanjan, Iran shows a probable risk for public health.

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