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Paper

Toxigenic Staphylococcus aureus in some animal-originated food products marketed in Turkey: presence and Public Health concerns

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Abstract

This study aimed to detect the presence of *Staphylococcus aureus* in some animal source food (ASF) including emulsified meat products (sausage and salami), dry fermented meat product (soudjouk), semi dry meat product (pastrami) and raw chicken meat. Sixty six (38.8%) of 170 samples were found to be positive for *S. aureus*. It was determined that *S. aureus* was found in 17 (56.6%) salami, 27 (54%) raw chicken meat, 9 (30%) soudjouk, 9 (30%) pastrami, 4 (13.3%) sausage samples. Staphylococcal enterotoxins (SEs) were identified in 5 out of 66 (7.5 %) isolates food matrices including 3 (4.5%) SEA, 2 (3.03%) SEC. The *sea* and *sec* genes were detected in 3 (4.5%) of 66 isolates. The results of this study highlight the need to provide suitable control strategies concerning production, sales, and storage to prevent the spread of enterotoxigenic *S. aureus* isolates in ASF. The key contribution of this study is its revelation of the presence of S. aureus in animal products sold in Turkish local markets, highlighting the potential public health risks associated with animal foods.

Keywords

Animal source foods, ELISA, multiplex PCR, toxigenic S. aureus.

Introduction

Staphylococcus aureus is a major bacterial human pathogen due to its ability to produce toxins and causes a wide variety of clinical symptoms(Ahmad-Mansour *et al*. 2021). Factors that increase the virulence of S. aureus are stated as enzymes such as coagulase hyaluronidase that are synthesized by the bacteria and spread in the host tissues and extracellular toxins that increase its pathogenicity. *S. aureus* strains can produce exfoliative toxins, hemolysins, epidermal cell differentiation inhibitors, Panton-Valentine leukocidin and extracellular protein toxins, and possess superantigenic activity toxins: toxic shock syndrome toxin-1 (TSST-1), and five major classical (SEA to SEE) and recently described new (SEG to SEJ, SEL to SEQ and SER to SET) types of enterotoxins (Oliveira *et al*. 2018). Staphylococcal enterotoxins (SEs) are a family of 25 protein exotoxins, described as highly stable and resistant to heat treatment, low pH and most of the proteolytic enzymes (Berry *et al*. 2022). SEA is the most frequently reported toxin in different foods and food poisoning outbreaks followed by SED, SEB and SEC (Le Loir *et al*. 2003, Soriano *et al*. 2002). Staphylococcal food poisoning symptoms appear in a few hours (2-8 h) after the ingestion of toxic dose (ranging from 20 ng to 1 µg) of SEs through food (Balaban and Rasooly, 2000). Symptoms include violent vomiting, nausea, abdominal cramps with or without diarrhea and are self-limiting within 24-48 h (Le Loir *et al*. 2003). TSST-1 is the major staphylococcal exotoxin, responsible for toxic shock syndrome involving fever, skin rash, diarrhea and hypotension symptoms. TSST-1 producing *S. aureus* strains were isolated from clinical samples of humans, animals and foods such as dairy, meat and RTE products (Cha *et al*. 2006, Oh *et al*. 2007).

Protein-rich foods such as milk, dairy products, meat and meat products and ready-to-eat (RTE) products are the

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most responsible for Staphylococcal food poisoning (Pelisser *et al*. 2009; Yildirim et al. 2017). ASFs play a crucial role as a source for common foodborne pathogens and their toxins during processing, preparation, packaging, grinding, and storage. ASFs are well-known reservoirs for *S. aureus* that have become an important global public health concern (Rortana *et al*. 2021, Wang *et al*. 2017; Yildirim *et al*. 2017). It is reported that miscellaneous risk factors such as improper environmental conditions for food preparation in retail markets, inadequate cleaning and sanitation practices, cross-contamination from contaminated materials and food handlers contribute to *S. aureus* contamination in foods (Al-Bahry *et al*. 2014, Mahyudin *et al*. 2019, Syne *et al*. 2013, Yildirim *et al*. 2017). To determine the contamination sources of *toxigenic S. aureus* in ASF is of utmost importance for preventing public health. Therefore, the present study was conducted i) to determine the prevalence of *S. aureus* in ASF, ii) to investigate the ability of the isolates to produce SEs and iii) to detect staphylococcal enterotoxin (sea to see) and tst genes in the isolates.

Materials and Methods

Sampling

A total of 170 ASF samples, including emulsified meat products(sausage, n=30 and salami, n=30), fermented meat products (soudjouk, n=30), ready-to-eat semi dry meat products (pastrami, n=30) and raw chicken meat (n=50), were collected from local markets in Kayseri, Turkey.

All samples were transported in a cool box from their place of collection to the laboratory and analyzed within 1-2 h.

Isolation of S. aureus

Twenty five grams of each sample was examined for Coagulase Positive Staphylococci (CPS) according to ISO 6888-1 (2003). Up to five presumptive coagulase positive Staphylococci (CPS) colonies (black to dark grey, smooth, convex, well-defined contours, surrounded or not by a dull halo) were subcultivated on blood agar for biochemical confirmation tests (Gram staining, coagulase test, catalase activity, oxidase reactions, DNase test).

Molecular identification of S. aureus isolates

Total genomic DNA was extracted from overnight-grown (at 35°C) cultures in Brain Heart Infusion Broth (Acumedia 7116A) using the InstaGene DNA Purification Matrix (Bio-Rad Laboratories, Hercules, CA). Amplification of *nuc* gene was performed according to described previous study by Cremonesi *et al*. (2005). In PCR reaction mixture the final volume 50 µL included 5 µL of template DNA, 10x PCR Buffer A (Vivantis, Malaysia), 1.5 mM $MgCl_2$ (Vivantis, Malaysia), 1.5 U Taq polymerase (Vivantis, Malaysia), 200 µM dNTP mix (Vivantis, Malaysia), and 30 mM of each primer (NUC-F166 and NUC-R565). The amplification was performed as follows: initial denaturation at 94°C for 5 min followed by a 30 cycles phase at 94°C for 1 min, annealing at 56°C for 1 min, extension phase at 68°C for 1 and final extension step at 72° C for 7 min (Techne TC-512, Keison Products, Chelmsford, UK)

Detection of tst gene and se genes

Two sets of primer mixes were prepared for the detection of *se* genes. The primer sequences used in the PCRs are described in Table 1. Multiplex primer set A and set B and PCR conditions were carried out according to Mehrotræ*t al*. (2000) and Sharma *et al*. (2000), respectively. Multiplex primer set A contained 10X reaction buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl); 200 mM dNTPs; 4 mM MgCl2; 5uL of template 1 U of Taq DNA polymerase, and 50 pmol for *tst* gene primers. The following thermal cycling profile was used: Initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 2 min, annealing at 57°C for 2 min, extension at 72°C for 1 min; final extension at 72°C for 7 min. Multiplex primer set B included the same components as in set A except for the primers, which were used at 20 pmol each one (for *sea-see* genes). The PCR amplification consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, a next step at 50 °C for 30 s and a 72 °C phase for 30 s. Final extension step was performed at 72 °C for 2 min.

All the amplified PCR products were determined by electrophoresis in 1.5% agarose gel in 0.5 X TBE buffer at 100 V for 40 min (EC250-90, Thermo, USA) and inspected visually under a UV transilluminator (Vilber Lourmat, Marne La Vallee, France).

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Gene	Primers	Oligonucleotide sequence (5'-3')	Size of amplified product (bp)	Reference
nuc	Nuc-F166	AGT TCA GCA AAT GCA TCA CA	400	Cremonesi et al. (2005)
	Nuc-R565	TAG CCA AGC CTT GAC GAA CT		
tst	GTSSTR-1	ACCCCTGTTCCCTTATCATC	326	Mehrotra *et al*. (2000)
	GTSSTR-2	TTTTCAGTATTTGTAACGCC		
SAU	(Primer Universal F)	TGTATGTATGGAGGTGTAAC	-	Sharma *et al*. (2000)
sea	SEA-R	ATTAACCGAAGGTTCTGT	270	
seb	SEB-R	ATAGTGACGAGTTAGGTA	165	
sec	SEC-R	AATTGTGTTTCTTTTATTTTCATAA	102	
sed	SED-R	TTCGGGAAAATCACCCTTAA	306	
see	SEE-R	GCCAAAGCTGTCTGAG	213	

Table I. Primers used for the detection of S. aureus and toxin genes.

Determination of Staphylococcal enterotoxins (SEs)

Enterotoxin production ability of *S. aureus* isolates was determined using Enzyme-Linked Immune Sorbent Assay (ELISA) technique (Thermo, Finland) with Ridascreen® SET A,B,C,D,E, r-biopharm (Germany, Art.no: R4101). The measurement was made photometrically at 450 nm on ELISA Reader (Thermo, USA).

Detection of SE's by ELISA

To determine enterotoxin production, initially, the isolates were incubated in 10 mL of Brain Heart Infusion Broth (BHI) (Oxoid, UK) for 24 hours at 37 °C. Then, the bacterial suspension was centrifuged at 3500 g for 5 minutes. The supernatant was used for ELISA. One-hundred microliters standard solutions and samples were inoculated in sepate wells and incubated at 37°C for 60 min in the dark. The liquid was discarded and the wells were washed four times with 300 µL wash buffer. Then, 100 µL of conjugate I was added in each well and incubated for 60 min at 37°C in the dark. The wells were again washed with 300 µL wash buffer for four times. After that, 100 µL of conjugate II was supplemented and incubated for 30 min at 37°C in the dark. The wells were washed again. To each well, 100 µL of substrate/chromogen was added and incubated for 30 min at 37°C in the dark. Then 100 µL of the stop reagent was put to each well and mixed and the opticial density (OD) was measured at 450 nm in ELISA reader (thermo, USA).The samples tested were assessed with regard to the Rida Soft Win computer program prepared by R-Biopharm. The cut-off value was used for evaluating the results as negative or positive and was calculated by adding 0.15 to the OD value of the negative control. The OD value of the samples, equal to or greater than the cut-off value, were considered positive.

Results

S. aureus were found in 66 (38.8%) of 170 samples analyzed: from 17 (56.6%) of the salami samples, from 27 (54%) of raw chicken meat, from 9(30%) of soudjouk, from 9 (30%) of pastrami and from 4 (13.3%) of the sausage.*S. aureus* counts in positive samples ranged 10^2-10^8 cfu/g giving an overall incidence of 38.8% which 33 (50%) were contaminated with ≥ 10^5 cfu/g (Table 2). In detail, *S. aureus* counts were determined above 10^5 cfu/g in16 (59.2%) raw chicken meat, 9 (52.9%) salami, 4 (44.4%) soudjouk, 3 (33.3%) pastrami and 1 (25%) sausage samples. SEs were detected in 5 (7.5 %) of 66 isolates by ELISA: SEA was determined in 3 (60%) isolates [2 from raw chicken meat and one from soudjouk samples] and SEC was detected in 2 (40%) isolates from raw chicken meat samples (Table 2). However, only 3 (4.5%) of 66 isolates contained SEs genes of which 2 (66.6%) were *sea* genes (from raw chicken meat and sausage samples), and one (33.3%) was *sec* genes (from raw chicken meat samples) (Table 2, Fig 1-3). Additionaly *S. aureus* counts of all SEs positive isolates were foundbetween 1 × 10^6 and 1 × 10^8 cfu/g. (Table 2).

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Samples	Number of *S. aureus* positive samples (%)	Distribution of *S. aureus* count (CFU/g)			Distribution of detected SE's (%)		Distribution of detected se genes (%)	
		$10^2 - 10^4$	$10^5 - 10^6$	$10^6 - 10^8$	SEA	SEC	*sea*	*sec*
Salami (n=30)	17 (56.6%)	8 (47.0%)	6 (35.2%)	3 (17.6%)	-	-		
Raw chicken meat (n=50)	27 (54%)	11 (40.7%)	9 (33.3%)	7(25.9%)	2 (7.4%)*	2 (7.4%)*	1(3.7%)	1 (3.7%)
Soujouk (n=30)	9 (30%)	5 (55.5%)	2 (22.2%)	2 (22.2%)	1 (11.1%)*	-	1(11 %)	
Pastrami (n=30)	9 (30%)	6 (66.6%)	3(33.3%)	-	-	-		
Sausage (n=30)	4 (13.3%)	3 (75%)	1(25%)	-	-	-		
Total (n=170)	66 (38.8%)	33 (50%)	21 (31.8 %)	12 (18.1%)	3 (4.5%)	2 (3.03%)	2 (3.03%)	1 (1.5%)

Table II. The distribution of S. aureus in the samples (CFU/g).

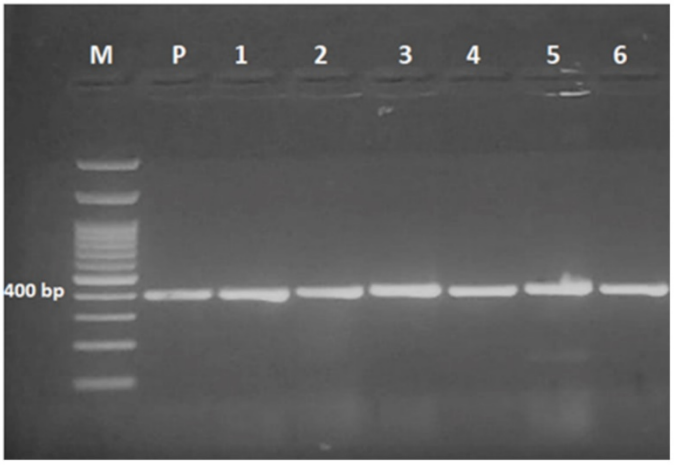

Figure 1. PCR product obtained with Nuc-F166/ Nuc-R565 primers:Lane M, molecular weight marker (Gene RulerTM 100 bp plus DNA Ladder, Thermo Scientific); P: positive control (S. aureus ATCC 29213, 400 bp), line 1-6: nuc gene positive samples.

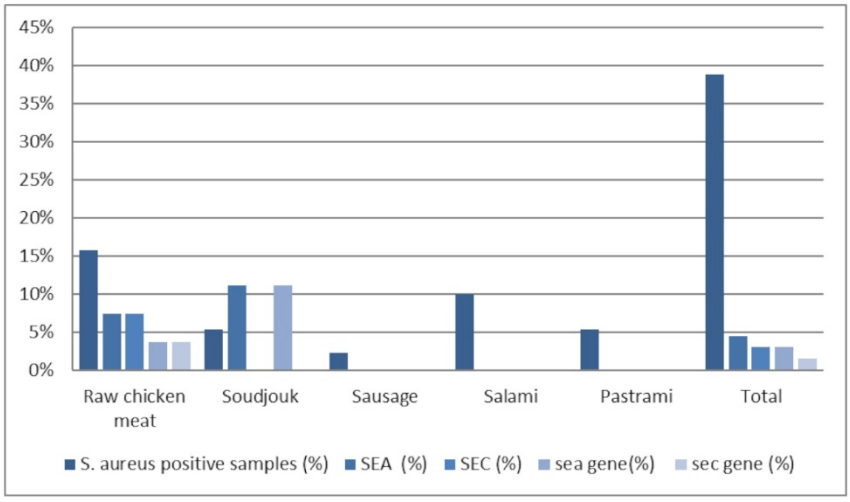

Figure 2. Presence of S. aureus in samples and SEs and se genes profiles in S. aureus isolates.

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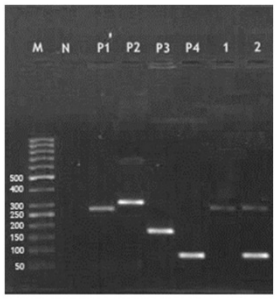

Figure 3. mPCR results for of enterotoxin A and C genes from S. aureus isolates: Lane M, molecular weight marker (Gene RulerTM 50 bp DNA Ladder Plus, Thermo Scientific), lane N: Negative control (H2O), Lane P1: positive control for sea (S. aureus ATCC 29231, 27270 bp), lane P2: positive control for sed (S. aureus NCTC 10652, 306 bp), lane P3: positive control for seb (S. aureus NCTC 10654, 165 bp), lane P4: positive control for sec (S. aureus, NCTC 10655, 69 bp), Lane 1: sea gene positive, obtained from soudjouk isolates, lane 2: sea and sec gene positive, obtained from raw chicken meat isolates.

Discussion

In this study, 38.8% of the 170 analyzed samples were found to contaminated with*S. aureus.* In earlier studies, *S. aureus* contamination rates were reported ranging from 13.7% to 79% obtained in meat products (Aydin *et al*. 2011, Normanno *et al*. 2005, Pelisser *et al*. 2009).We noted that *S. aureus* counts were also determined above 10^5 cfu/g in 9 (52.9%) salami, 4 (44.4%) soudjouk, 3 (33.3%) pastrami and 1 (25%) sausage samples. Similar to the results of this study, Morshdy et al. (2018) reported the mean *S. aureus* number as 4.3×10^2 cfu/g in 8% of the sausage samples they examined; on the other hand, Hassanina et al (2018), stated that 63.33% of the sausage samples they examined were contaminated with *S. aureus* with an average number of 5.96×10^3 cfu/g. Emulsified meat products are made by a similar process which includes smoking and/or cooking. The growth of pathogens and staphylococcal enterotoxin production in this type of product might be inhibited by these processing properties. However *S. aureus* distribution and counts were found to be high in salami samples, one of the emulsified meat product varieties examined in this study, may be because the analyzed salami samples were purchased in slice portions from the open sale in the markets. Previous studies reported that equipment (knives, cutting boards, food contact surfaces, e.g.) and food handlers serve as vehicles transferring the *S. aureus* to products during meat slicing (Syne *et al*. 2013, Yildirim *et al*. 2017). Our results also showed that 30% of the other sliced meat product- pastrami- samples were found to be positive for *S. aureus* and 33.3% of them were found to exceed 10^5 cfu/g. Earlier studies reported the prevalence of*S. aureus* ranging from 2.2% to 100% in pastrami (Elmali et al., 2007; Gungor et al.,2021; Yildirim et al., 2017). However, Elmalı et al. (2007) reported that *S. aureus* was detected as $<10^2$ cfu/g in all of the samples examined, while Yıldırım et al. reported that *S. aureus* was detected as $>10^2$ cfu/g in only 2.6% of the positive samples. Considering the higher*S. aureus* cell numbers was in salami samples than pastrami samples, it could be related to physicochemical properties of products. Althought pastrami is an uncooked RTE meat product, it's semi dry and covered fenugreek paste which has an antimicrobial effect against some bacteria including *S. aureus* (Yetim et. al., 2006).

In this study, *S. aureus* has been isolated from 30% of dry-fermented meat product samples (soudjouk) and 44.4% of the samples were harboured *S. aureus* counts greater than 10^5 cfu/g where enterotoxin has also been detected. Similar to the results of this study, Samappito et al. reported that they determined the average number of *S. aureus* as 5.7×10^5 cfu/g in dry fermented sausage samples called 'Mhom' in their study in Thailand in 2011. The growth of pathogens in the fermented meat products are prevented by the addition of starter cultures, pH reduction and/or dryness. Turkish soudjouk is traditionally made from raw ruminant meat via a natural fermentation (Calicioglu et. al., 2001). Akkaya et al. (2014) reported that the initial numbers of lactic acid bacteria was important for the rapid decline in the acidity which was considered to be the main factor in the inhibition of *S. aureus* growth in soudjouk. The using natural lactic acid bacteria in soudjouk production might cause a delay in the rapid reduction of the pH because natural lactic acid bacteria could not reach enough population number in soudjouk compared to starter cultures (Kaban ve Kaya, 2006). According to label information, soudjouk samples analyzed in this study were fermented using natural lactic acid bacteria. It is estimated that the use of a natural lactic acid bacteria caused a delay in the rapid increase of the acidity and allowed *S. aureus* growth and produced enterotoxins during the the initial stage of fermentation.

Raw chicken meat also showed a high prevalence of contamination (54%) and 16 out of 50 samples (59.2%) were found to contaminate with $\geq 10^5$ cfu/g. Prevalence rates reported by other studies in chicken meat were variable: 40-60% (Alvarez-Astorga *et al*. 2002); 65.8% (Kitai *et al*. 2005) and 55% (Guven *et al*. 2010). Also, previous studies indicated that the cross-contamination from environments (such as human, poultry processing plant, water, or

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equipment) appears to be a greater source of *S. aureus* contamination of raw chicken meat compare to direct animal transmission (Al-Bahry *et al*. 2014, Hanning *et al*. 2012, Kitai *et al*. 2005).

In this study 3 isolates produced SEA and 2 isolates produced SEC, according to ELISA results; however,*sea* and *sec* genes, respectively, were detected in only 2 and 1 of these isolates by PCR. These differences might be related to immunological cross-reactions among polyclonal antibodies prepared against SEs, due to the presence of similar epitopes in the proteins (Munson et al., 1998; Nájera-Sánchez et al., 2003 Aydin et al. 2011). Munson et al. (1998) reported that SEG have epitopes in common with SEC. They also suggested SEG is similar to SEB (approximately 39% amino acid identity), whereas SEI is closely related to SEA, SEE, and SED with amino acid identities ranging from 26 to 28%.

This study indicates that among enterotoxigenic *S. aureus* isolates, SEC producers recovered from raw chicken meat samples, while SEA producers from raw chicken meat and soudjouk sample. Classical SEs (SEA to SEE) have been reported to cause 95% of staphylococcal food poisoning, with the most common enterotoxin SEA (Soriano *et al*. 2002, Oh *et al*. 2007). Different frequencies of enterotoxigenic *S. aureus* detection in raw chicken meat was reported by previous studies as: 5.9% (Abdalrahman *et al*. 2015), 21.7% (Kitai *et al*. 2005) and 63.6% (Guven *et al*. 2010). Similar to the results of this study, Guven *et al*. (2010) reported that the highest prevalence and counts of *S. aureus* were found in salami, poultry, and soudjouk samples and from which only poultry and soudjouk isolates containing SEs.

In this study, meat products containing various levels of salt and nitrite were analyzed and they are allowed to be used in the Communique of Meat and Meat Products of the Turkish Food Codex. Previous researchers have reported that *S. aureus* is tolerant to salt and nitrite which has also been confirmed in this study (Bang *et al*. 2008, Correia *et al*. 2014, Gonzalez-Fandos *et al*. 1999, Lin *et al*. 2018). Regassa *et al*. (1993) also reported that NaCl stress could be decreased *sec* gene expression. Previous studies also suggest that storage temperature, and mild NaCl stress might affect to *sea* gene expression in meat products (Tsutsuura *et al*. 2013, Zeaki *et al*. 2014, 2015). It might be the elucidation for not detecting SEs in *S. aureus* positive pastrami, salami and sausages samples which contained various levels of salt in our study. It was noteworthy that although five of the isolates contained SEs, three of them harbored *se* genes. In detail, 3.7% of raw chicken meat and 11.1% of soudjouk isolates had *sea* genes while 3.7% of raw chicken meat isolate had *sec* genes.

Conclusion

In conclusion, detecting *S. aureus* and its enterotoxins in animal products sold in local markets highlights the potential public health risks. Given the ability of these pathogens and their toxins to cause a wide range of enterotoxemia, it is essential to implement effective monitoring and control measures to prevent the spread of these environmental contaminants in the food supply chain. Caretakers and food safety professionals should also take appropriate precautions when handling and preparing animal products to minimize the danger of foodborne illnesses. Animal products need to be ensured as safe by remaining vigilant and proactive in addressing these risks via hazard analysis within the frame of comprehensive food safety management systems.

Conflict of interest

The authors have declared that there is no conflict of interest in this study

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