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Prevalence of *Staphylococcus aureus* Isolated From Various Foods of Animal Origin in Kırşehir, Turkey and Their Enterotoxigenicity

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ABSTRACT

The aim of this study was to detect *Staphylococcus aureus* contamination to different types of animal origin foods collected in the Kırşehir province of Turkey and to examine their enterotoxin production ability. Out of 120 food samples 38 suspected colonies were obtained and 23 of them were identified as *S. aureus* by biochemical and molecular analyses. Other species detected were *S. chromogenes*, *S. cohnii* ssp. *cohnii*, *S. hominis*, *S. lentus*, *S. warneri*, and *S. xylosus*. The isolates were also analysed with regard to carry *mecA* gene. None of them was found to have *mecA* gene indicating susceptibility to methicillin. To determine the enterotoxigenic ability of the isolates phenotypically, reversed-passive-latex-agglutination test against SEA-SED was used. Six out of 23 *S. aureus* isolates were determined to produce SEA, SEC and SED. Three of them had only one enterotoxin production, whereas others had SEA and SED production together. The results of phenotypic analyses were confirmed by PCR based examination. None of the coagulase-negative staphylococci were found to be enterotoxigenic by both phenotypical and PCR-based analyses. In conclusion, enterotoxigenic *S. aureus* is a risk in foods of animal origin in Kırşehir and its counties.

Introduction

Staphylococcus species are frequently found in foods and *S. aureus* is the one that causes food poisoning (Roberts et al., 2005). Foodborne outbreaks due to *S. aureus* are widespread in the world, regardless of the country level of development. Prevalent strains and enterotoxins vary by geographical region (Hennekinne et al., 2012). According to Aydin et al. (2011), *S. aureus* contaminations of foods were 13.8% in Marmara Region of Turkey during the period of July 2007-2008. In the European Union, outbreaks arising from bacterial toxins (produced by *Bacillus*, *Clostridium* and *Staphylococcus* species) were 16.1% following virus and *Salmonella* outbreaks, and 393 food-borne outbreaks were caused by staphylococcal toxins in 2014 (EFSA, 2014). Out of 1229 foodborne outbreaks of toxigenic bacteria (*Bacillus cereus*, *Clostridium perfringens* and *S. aureus*) 459 were *S. aureus* outbreaks in the US during 1998-2008 (Bennet et al., 2013). To be a public health hazard via infection, food poisoning and outbreaks is one dimension of this pathogen's significance. The other dimension consists of economic loss, worker's health and safety in food and agriculture industry. Moreover, antimicrobial resistance of *S. aureus* is making the pathogen more hazardous, especially methicillin resistance. In recent years, methicillin resistant *S. aureus* (MRSA) strains were reported not only in hospital infections, but also in livestock related infections and in foods such as raw milk and meat (Springer et al., 2009; Türkyılmaz et al., 2010; Fluit, 2012). In this case, foods constitute a reservoir for such strains.

One of the contamination ways of *S. aureus* to foods is the food handlers who carry the bacteria on their skins and mucosal tissues. Animals having sub-clinical mastitis due to *S. aureus* are another source for transmission, especially in dairy and livestock farming. Besides, foods can be contaminated by air, dust and surfaces in contact with foods (Argudín et al., 2010). Contaminated foods are often milk and products prepared with milk, such as cream and pudding, meat, fish and delicatessen (Schelin et al., 2011).

Diseases caused by *S. aureus* vary between simple skin infections to severe systemic infections in humans. Food poisoning is among them and staphylococcal enterotoxins are the causing agent, so named as staphylococcal intoxication. Symptoms of the intoxication occur 1-8 hour after the ingestion of the contaminated food and are nausea, severe vomiting, abdominal cramps and diarrhea (Kadariya et al., 2014).Consumer may not be aware of contaminated food, because it is not necessary that the contaminated food has a sensory defect. The bacterial growth level, causing intoxication is $>10^5$cfu/g

and enterotoxin level is <1 µg/g of food (Pinchuk et al., 2010).

Staphylococcal enterotoxins (SEs) are produced in logarithmic growth phase or entry to stationary phase of bacteria on food. They are stable to food preservation techniques, due to their low molecular weight protein structure, resistance to heat or proteolytic enzymes (Argudínet al., 2010). SEs together with staphylococcal enterotoxin-like toxins (SE*l*) are divided more than 20 different serological groups named SEA to SEIV, among which SEA, SEB, SEC and SED are the most prevalent in staphylococcal foodborne outbreaks (Hennekinne et al., 2010). Besides *S. aureus*, coagulase negative staphylococci (CNS) were also determined to produce SEs (Zell et al., 2008; Bertelloni et al., 2015).

The aim of this study was to reveal the *S. aureus* contamination in foods of animal origin sold in Kırşehir province of Turkey and to characterize the enterotoxin production of the isolates. For this aim, food isolates were identified, and enterotoxin production was detected by immunological tests and PCR analyses. Furthermore, whether the isolates had *mecA* gene was also investigated. To our knowledge, this is the first study reporting *S. aureus* contamination and their enterotoxin production in foods collected in Kırşehir. The reason to conduct this study in Kırşehir was the potential of dairy and livestock farming of the province.

Materials and Methods

Bacterial Isolations and Identifications

120 food samples were collected from doner kebap restaurants, retail markets, butchers and barns in Kırşehir and its counties during July 2012-March 2013. The food groups were raw milk (51 samples), local cheese (5 samples), raw meat or offal product (21 samples), raw poultry meat or giblet (23 samples) and chicken doner kebap (20 samples). The small sample size and variation in food groups were due to the difference of the counties market potential. 25 g or ml of food sample was mixed with 225 ml of Buffered Peptone Water (Oxoid, Basingstoke, England) in a bench top stomacher (Mayo, Italy) and incubated at 37°C for overnight. Then it was transferred to Giollotti-Cantoni Broth (Merck, Germany) and then Baired Parker Agar (Oxoid, Basingstoke, England) containing egg yolk tellurite (Oxoid, UK), each of which was incubated at 37°C for overnight. Suspected colonies of *S. aureus* were analyzed under microscope by using Gram staining and the slide coagulase test was done by using rabbit plasma. API Staph (bioMérieux, France) test kits were used for biochemical identification of the isolates. The isolates were stored in Brain Heart Infusion Broth (BHI, Merck, Germany) containing 15% glycerol at -80°C.

DNA Isolation

DNA isolation of the isolates was done as described MLST.net (http://saureus.mlst.net/misc/info.asp). Briefly, a fresh culture of each isolate on BHI Agar (Merck, Germany) was mixed with the lysis solution (0.01 M EDTA, 0.01 M Tris, 25 units/ml Lysostaphin and 250 units/ml Lysozyme) and incubated at 37°C in the water bath (JSR, Korea) for 30 min. Then the temperature increased to 95°C and samples were boiled for 10 min. Immediately after, they were cooled down with ice. Phenol: chloroform: isoamylalcohol (25:24:1) extraction and ethanol precipitation were applied by 10000 g for 20 min centrifugation. The raw DNA of each isolate was kept in molecular grade water at -80°C. This raw DNA was used as the template in all PCR analyses.

nuc *and* mecA *Gene Region Analyses*

The analyses of all isolates *nuc* and *mecA* gene regions were done by using a multiplex PCR assay as described in Costa et al. (2004). 5 µl raw DNA was added to 20 µl PCR mixture containing 12.5 µl 2×master mix (Thermo Scientific, USA) and 0.2 µM end concentration of each primer (Table 1). Thermocycling was carried out using Cleaver Scientific GTC96S (UK) gradient thermalcycler with the following conditions: initial denaturation at 95°C / 8 min followed by 30 cycles of 94°C / 1 min, 55°C / 30 s, and 72°C / 90 s, the final step at 72°C / 1min and at 4°C / limitless. Amplified fragments were run on agarose gels (1.5% agarose in 1×Tris-borate EDTA) at 70 V for 40 min. The gel was stained with ethidium bromide (Thermo Scientific, USA) and visualized under UV illumination (DNR, Bio-Imaging Systems, MiniLumi, Israel). *S. aureus* NCTC 10442 was used as positive control.

Reversed-Passive-Latex Agglutination Assays of SEs

SEA, SEB, SEC and SED production ability were analyzed by using SET-RPLA test kits (Oxoid, Basingstoke, England). All isolates were incubated in BHI broth (Merck, Germany) at 37°C for 24 hand broth cultures were centrifuged at 4°C and 900g for 20 min. Supernatants were used to test enterotoxin production in V-shaped well microtiter plates as recommended by the manufacturer's instructions.

Detection of Genes Encoding SEs

For analysing whether the isolates have *sea*, *seb*, *sec*, *sed* or *see* genes, multiplex PCR was applied on all isolates according to Becker et al. (1998). The reaction mixture contained 5 µl raw DNA and 45 µl PCR mixture having 25 µl 2 × master mix (Thermo Scientific, USA) and 0.5 µM end concentration of each primer (Table 1). Thermocycling conditions were initial denaturation at 95°C / 2 min followed by 30 cycles of denaturation at 95°C /1 min, 55°C / 1 min, and 72°C / 2 min, the final step at 72°C / 5 min and at 4°C / limitless. Amplified fragments were run on agarose gels (2% agarose in 1 ×Tris-borate EDTA) at 100 V for 2 h. The gel was stained with ethidium bromide (Thermo Scientific, USA) and visualised under UV illumination (DNR, Bio-Imaging Systems, MiniLumi, Israel).*S. aureus* NCTC 10652 (*sea*), NCTC 10654 (*seb*), NCTC 10655 (*sec*) and NCTC 10656 (*sed*) were used as positive controls.

Table 1 List of primers used

Gene	Primers	Primer sequences (5'-3')	Size (bp)	Reference
nuc	nuc-1/2	GCGATTGATGGTGATACGGTT / AGCCAAGCCTTGACGAACTAAAGC	267	Brakstad et al. (1992)
mecA	mecA-3/4	AAAATCGATGGTAAAGGTTGGC / AGTTCTGCAGTACCGGATTTGC	533	Brakstad et al. (1992)
sea	SEA3/4	CCTTTGGAAACGGTTAAAACG / TCTGAACCTTCCCATCAAAAAC	127	Omoe et al. (2002)
seb	SEB1/4	TCGCATCAAACTGACAAACG / GCAGGTACTCTATAAGTGCCTGC	477	Omoe et al. (2002)
sec	SEC3/4	CTCAAGAACTAGACATAAAAGCTAGG / TCAAAATCGGATTAACATTATCC	271	Omoe et al. (2002)
sed	SED3/4	CTAGTTTGGTAATATCTCCTTTAAACG / TTAATGCTATATCTTATAGGGTAAACATC	319	Omoe et al. (2002)
see	SEE3/2	CAGTACCTATAGATAAAGTTAAAACAAGC / TAACTTACCGTGGACCCTTC	178	Omoe et al. (2002)

Results and Discussion

S. aureus is an extensively studied pathogenic bacteria in the world due to diseases and outbreaks caused by the bacterium. It has appeared in the literature that many foodborne outbreaks arose from enterotoxigenic *S. aureus*. Pu et al. (2011) reported the prevalence of enterotoxigenic *S. aureus* in various foods that 59.8% in Italy, 62% in Korea, 74% in Poland, 69% in Portuguese, 30.5% in France and 39.2% in Slovak Republic. In another study, 52 *S. aureus* strains of which ST6 was the dominant clone were found to be associated with 11 outbreaks in Shenzhen, China between 2006 and 2009 (Yan et al., 2012). *S. aureus* was obtained in 73 of 693 food samples (10.5%) in Alberta, Canada during January 2007-December 2010 (Crago et al., 2012). In this study, out of 120 animal origin food samples, 38 presumptive *S. aureus* isolates were obtained. However, biochemical identifications and *nuc* gene amplifications demonstrated that 23 of the isolates were *S. aureus* (19.2%) (Table 2). In all counties of Kırşehir, *S. aurues* contamination was determined. Considering that Kırşehir is a small province (225562 inhabitants in 2015) and animal husbandry is one of the main sources of income of the inhabitants, *S. aureus* is a risk in all counties of Kırşehir according to these results. *S. aureus* contamination in various foods in Turkey was reported as 57.3% in the Kayseri province (Ertaset al., 2010), 33.4% in Kütahya and Eskişehir region, (Guvenet al., 2010), 13.8% in the Marmara region (Aydin et al., 2011), 6% in Ankara province (Can and Çelik, 2012), 52.5% in Samsun province (Gücükoğlu et al., 2012). The variation of these results was owing to the fact that these studies were conducted on different sample sizes and different food groups. However, contamination rate was also be varied due the different geographical regions of Turkey.

The most contaminated food samples have belong to a raw milk group (31.4% within-group) followed by raw meat group (23.8% within-group), chicken doner kebap (5% within-group) and raw poultry group (4.3% within-group). Milk samples were of bovine origin. Raw bovine milk is a frequently contaminated food source, since *S. aureus* is a common cause of bovine mastitis (Kadariya et al., 2014). The raw meat group was the second source of contamination in this study. In contrast, Guven et al. (2010) reported that the meat products contamination rate (48.7%) was significantly higher than milk and dairy products (23.2%) in Eskişehir and Kütahya region of Turkey. Although the rate of raw milk sample contamination was similar to their study, the controversy was originated from sample region. Another study was reported even more contamination rate (75%) in raw milk in Samsun province (Gücükoğlu et al., 2012). The high contamination rates of raw milk samples in Turkey reveals the significance of this pathogen in dairy animals. The results of this study were indicating more of a bovine mastitis prevalence in Kırşehir. Because *S. aureus* is more prevalent in milk from cows with mastitis in comparison with milk from healthy cows (Rall et al, 2014). The results of the poultry sample group exhibited that it was the least contaminated group comparing with other food groups. In the US, meat or poultry dishes were reported to be more related with foodborne outbreaks of *S. aureus.* Outbreaks caused by *S. aureus* from 1998 to 2008 were due to 10% of beef, 14% of poultry dishes (Bennet et al., 2013). Cheese, offal or giblet samples in this study were not found to be contaminated with *S. aureus.* On the other hand, *S. aureus* isolations from cheese and giblets were existed in the literature. Twenty one positive samples were detected out of 105 cheese samples in Italy (Carfora et al., 2015). On the other hand, 9% of contamination was reported for cheese products collected in Ankara, Turkey (Can and Çelik, 2012). Nine *S. aureus* isolates out of 30 chicken giblets were identified by Gundogan et al. (2005). Comparing with these researches, S. *aureus* contamination must be further analysed for cheese, offal and giblet samples in Kırşehir.

Food isolates of CNS are also a research interest. Although they are not associated with foodborne outbreaks, these strains are important to constitute a reservoir for antimicrobial resistance, virulence and enterotoxin genes. Moreover, enterotoxigenic strains are also known. In this study, species detected other than *S. aureus* were *S. chromogenes*, *S. cohnii* ssp. *cohnii*, *S.*

hominis, *S. lentus*, *S. warneri*, *S. xylosus* and an undetected isolate (Table 2). Bertelloni et al. (2015) isolated 74 CNS out of 120 bovine bulk tank milk samples, in which *S. xylosus*, *S. chromogenes*, *S. sciuri* and *S. hominis* were the most prevalent species. The results of this study were parallel to Bertelloni et al. (2015) that *S. xylosus* was the species isolated with the highest prevalence (8 out of 14 CNS). In contrast, *S. xylosus* isolates were obtained from food samples other than raw milk.

The actual role in staphylococcal food poisoning belongs to staphylococcal enterotoxins produced during bacterial growth on the food. 393 food-borne outbreaks were caused by staphylococcal toxins in 2014 (EFSA, 2015). The most enterotoxigenic species is *S. aureus* among other staphylococcal species, and contamination rates and toxin production of the pathogen differ in its strains. Enterotoxigenic capacity of the isolates were also investigated in this study. Out of 23 *S. aureus* isolates, 6 (26%) were determined to have an enterotoxin production ability (Table 3) and corresponding genes *sec, sed* and *sea/sed* (Figure 1). In a study carried out with raw milk and cheese samples, it was exhibited that the rate of enterotoxigenic *S. aureus* was 64.8%, in which SEC was the most prevalent enterotoxin produced (Gundogan et al., 2005). In another study, Argudín et al. (2012) reported that 64 *S. aureus* isolates recovered from foods and food handlers were able to produce SE or SE*l*except four isolates. Furthermore, they obtained *sea* (38.7%), *seb* (12.9%), *sec* (16.1%), and *sed* (22.6%) as the most prevalent genes. The results of this study indicated that the most prevalent enterotoxins were found in the isolates and these findings were similar to those found in literature. Ertas et al. (2010) reported that *S. aureus* isolates from dairy desserts and sheep cheeses 16% were enretoxigenic and *sea, seb, sec* and *sed* were the genes detected. Also enterotoxigenic isolates were found to be 62.6% in the Marmara region of Turkey, and *sea* was the prevalent gene (Aydin et al., 2011). In another study, 25% of *S. aureus* isolates of cheese origin were reported to be SEC and SEC/SED producer (Can and Çelik, 2012).

Table 2 Identified species of the isolates.

Species	County*	Food Source	NI
S. aureus	Ak, Ap, B, C, K, M, Mu	Raw milk, raw meat, raw poultry, donerkebap	23
S. chromogenes	B	Raw milk	2
S. cohnii ssp. *cohnii*	K	Raw poultry	1
S. hominis	K	Giblet	1
S. lentus	Mu	Donerkebap	1
S. warneri	B	Raw milk	1
S. xylosus	Ak, K	Raw meat, offal, raw poultry, donerkebap	8

* Ak: Akçakent, Ap: Akpınar, B: Boztepe, C: Çiçekdağı, K: Kaman, M: City centre, Mu: Mucur, NI: Number of Isolate

Table 3 Enterotoxin producing isolates and their origins.

Isolate No.	County	Food Source	Enterotoxin
1112085	City centre	Raw milk	SEC
1112086	City centre	Raw milk	SED
0213113	Boztepe	Raw milk	SEA and SED
0213117	Boztepe	Raw milk	SEA and SED
0213118	Boztepe	Raw milk	SEA and SED
0213142	Akçakent	Raw milk	SEC

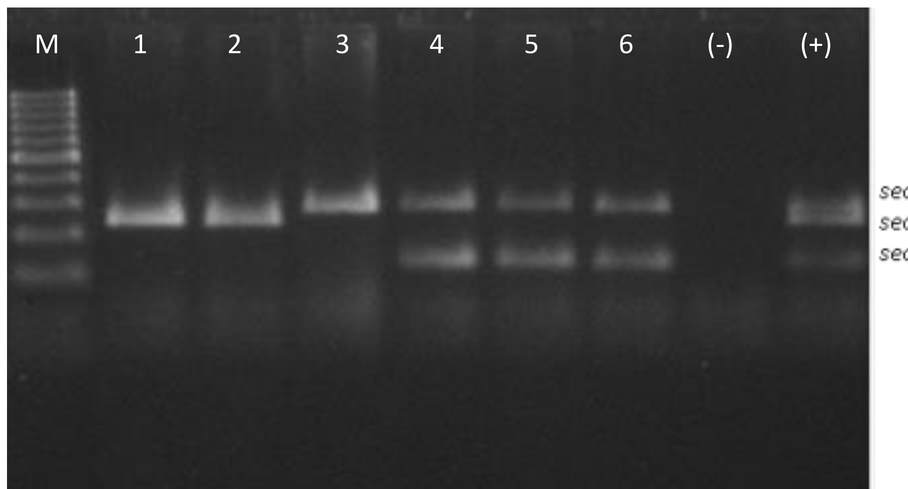

Figure 1 PCR results of enterotoxigenic isolates (1: 1112085; 2: 0213142; 3: 1112086; 4: 0213113; 5: 0213117; 6: 0213118; (-): molecular grade water; (+): S. *aureus* control strains)

On the other hand, now it is known that CNS has the ability to produce enterotoxins (Zell et al., 2008). Bertelloni et al. (2015) conducted a study with CNS isolated from bovine raw milk and obtained that out of 74 isolates 40 were enterotoxigenic. Thus, it was analysed whether the CNS isolates of this study had the enterotoxin production ability. However, CNS isolates of this study were not enterotoxigenic in terms of the tested enterotoxins.

Antibiotic resistance in *S. aureus* strains have been known since the 1930s. When it comes today, hospital or community acquired methicillin resistant *S. aureus* strains are seen widely in the world. Nowadays, MRSA strains were also reported from foods of animal origin (Gundogan et al., 2005; Can and Çelik, 2012; Fluit, 2012; Carfora et al., 2015). The isolates of this study did not exhibit *mecA* gene, but further study must be conducted with antimicrobial resistance profiles of *S. aureus* strains in order to clarify the situation.

Conclusion

In conclusion, enterotoxigenic *S. aureus* is a risk in foods of animal origin in Kırşehir and its counties. Raw milk is the most contaminated food source and enterotoxin producing strains are prevalent in raw milk. This study was a pilot study to investigate the enterotoxigenic *S. aureus* strains in foods, thus it should be extended with a larger size of sampling in order to reveal the risk with all aspects. Besides, the sanitary practices must be provided in both dairy farms and food producing facilities, and careful attention must be paid on animal hygiene to avoid mastitis.

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