



Detection of *Staphylococcus aureus* enterotoxins in sheep cheese and dairy desserts by multiplex PCR technique

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ABSTRACT

The aim of this study was to investigate the presence of *Staphylococcus aureus* (*S. aureus*) and staphylococcal enterotoxins (SEs) genes in sheep cheese and dairy dessert samples by multiplex PCR (mPCR) technique. A total of 150 samples were analyzed consisting of 50 dairy dessert samples and 100 sheep cheese. Coagulase positive staphylococci (CPS) were found in 86 (57.3%) out of 150 analyzed samples. *S. aureus* were isolated from 60 (60%), 26 (52%) of sheep cheese and from of dairy desserts, respectively. Five suspected colonies were tested from each sheep cheese and dairy dessert samples for phenotypic and genotypic characterizations. A total of 430 isolates from the 86 positive samples were investigated in this study. Eighty (18.6%) isolates were characterized as *S. aureus*. The enterotoxin genes (*sea*, *seb*, *sec*, *sed*) were found in 13 (3.02%) out of 80 isolates. From cheese isolates, *sea*, *seb* and *sed* were detected in 5 (1.6%), 2 (0.6%), 1 (0.3%), respectively. From dairy dessert isolates, *sea*, *sec* and *sed* were detected in 3 (2.3%), 1 (0.76%), 1 (0.76%), respectively. The presence of SEs was identified in 12 (2.8%) out of 80 isolates by using ELISA technique. It was determined that these SEs had a distribution of 7 (1.6%) SEA, 2 (0.46%) SEB, 1 (0.23%) SEC, and 2 (0.46%) SED. SEs were found in 7 (2.3%) cheese and 5 (3.8%) dairy dessert isolates. In conclusion, *S. aureus* and their SEs were found to be present in sheep cheese and dairy desserts in this study. It is emphasized that the presence of *S. aureus* and their SEs genes in sheep cheese and dairy desserts may be regarded as a potential risk for human health.

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1. Introduction

Staphylococcus aureus is one of the most common agents in bacterial food poisoning outbreaks (Loncarevic et al., 2005; Blaiotta et al., 2004; Pelisser et al., 2009). *S. aureus* produces different extra-cellular protein toxins and virulence factors, which enhance its pathogenicity due to their enterotoxins (Holeckova et al., 2002; Tkacikova et al., 2003; Akineden et al., 2008). There are several types of SEs (A, B, C, D, E, G, H, I, J, K, L, M, N, O, P, Q, and R) and the corresponding genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq*, *ser* and *seu*) have been described (Munson et al., 1998; Su and Wong, 1998; Carmo et al., 2002; Blaiotta et al., 2004; Loncarevic et al., 2005; Normanno et al., 2005; Pelisser et al., 2009). Type A and type D of SEs are especially responsible for foodborne poisoning (Pelisser et al., 2009; Hwang et al., 2007). SEs are thermostable which means that biological activity of toxins remains unchanged even after thermal processing of food and also resistant to gastrointestinal proteases such as pepsin. It can remain active after ingestion (Pelisser et al., 2009; Balaban and Rasooly, 2000; Adwan et al., 2005; Holeckova et al., 2002; Cremonesi et al., 2007). There are many factors affecting enterotoxin production in food such as cell count, salt

concentration, pH, temperature and presence of competitive flora (Pelisser et al., 2009; Balaban and Rasooly, 2000; Soriano et al., 2002; Sokari, 1991; Necidová et al., 2009; Cremonesi et al., 2007). SEs in dairy products such as raw milk and cheese have been correlated with food poisoning (Carmo et al., 2002; Rosec and Gigaud, 2002; Jorgensen et al., 2005; Le Loir et al., 2003; Brasca et al., 2005; Akineden et al., 2008). Although milk and milk products are frequently contaminated with *S. aureus*, dairy products are rarely involved in staphylococcal food poisoning. Also, bacteria counts of 10^5 – 10^6 CFU/g are required before their enterotoxins can cause food-related poisoning (Akineden et al., 2008; Pelisser et al., 2009; Necidová et al., 2009). Although the pasteurization kills *S. aureus* cells, thermostable SEs generally retain their biological activity, therefore, these toxins are important in the public health and food sectors (Evenson et al., 1988; Asao et al., 2003; Le Loir et al., 2003; Becker et al., 2007). Intoxication is resulting from the ingestion of food containing heat-stable enterotoxins, usually produced by this microorganism. Symptoms are characterized by nausea, vomiting, abdominal cramps and diarrhea (Jorgensen et al., 2005; Normanno et al., 2005; Holeckova et al., 2002; Loncarevic et al., 2005).

The detection of *S. aureus* and SEs in food is difficult (Monday and Bohach, 1999). Methods currently used for detection of SEs in food are Enzyme-Linked Immunosorbent Assay (ELISA), reversed passive latex agglutination (SET-RPLA), polymerase chain reaction (PCR) technique (Monday and Bohach, 1999; Loncarevic et al., 2005; Tkacikova et al., 2003; Cremonesi et al., 2007).

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The objective of this study was to investigate the presence of *S. aureus* and SEs in sheep cheese and dairy dessert samples by using mPCR technique for special emphasis on their enterotoxigenic potential.

2. Materials and methods

2.1. Materials

In this study, a total of 150 samples were collected from August to December 2009 in Kayseri in Turkey. Sheep cheese ($n=100$) and dairy desserts ($n=50$) were purchased from different markets and other open bazaars in Kayseri. The samples were immediately transported to the laboratory in a cool box and examined within 1–2 h of sampling for the presence of *S. aureus*.

A total of 50 dairy dessert samples were analyzed consisting of 10 keskul (milk pudding containing coconut), 10 tavuk gogsu (milk pudding containing chicken chest), 10 kazandibi (milk pudding slightly burned on the bottom), 10 supangle (milk pudding containing chocolate) and 10 sutlac (milk pudding containing rice).

2.2. Isolation media

Baird Parker Medium (BPM; Oxoid, CM 275) with 5% egg yolk and tellurite (Merck 1.03785.0001) was used for isolation of *S. aureus*.

2.3. Bacterial strain

Reference strains of *S. aureus* ATCC 29213 (SEA), *S. aureus* NCTC 10652 (SEA, SED), *S. aureus* NCTC 10654 (SEB), *S. aureus* NCTC 10655 (SEC) were used as positive controls in this study (provided by Gulhan T, Department of Microbiology, School of Veterinary Medicine, University of Yuzuncu Yil, Van, Turkey).

2.4. Primers

Five should be changed to six PCR primers, SA-U, SA-A, SA-B, SA-C, ENT-C, SA-D were used for mPCR assay, as described by Sharma et al. (2000).

2.5. Microbiological analyses

For *S. aureus* isolation, 10 g of each samples was added to 90 ml sterile peptone water and homogenized for 60–90 s in Stomacher Lab-Blender (Interscience). Serial dilutions of samples homogenates were surface plated on BPM (Oxoid, CM 275) with 5% egg yolk and tellurite (Merck 1.03785.0001). The plates were incubated at 37 °C for 24 h. Colonies with typical black appearance and surrounded by clear zone were enumerated as *S. aureus* (Pelisser et al., 2009; Loncarevic et al., 2005). In this study, five suspected colonies from each sheep cheese and dairy dessert samples were tested phenotypically and genotypically.

2.6. Phenotypical characterization of isolates

Five colonies that grew in BPM were transferred to blood agar (Merck, 1.10886.0500). Colonies which grew in blood agar were tested for Gram coloration, coagulase, catalase, oxidase, and urease (Pelisser et al., 2009).

2.7. DNA extraction

Total genomic DNA was extracted from overnight-grown at 35 °C CPS cultures in Brain Heart Infusion broth (Acumedia 7116A). DNA was isolated by using the Genomic DNA Purification Kit (Axygen, Bioscience, USA).

Table 1

The distribution of *S. aureus* in sheep cheese and dairy desserts (CFU/g).

Samples	Number of samples	Number of <i>S. aureus</i> positive	Distribution of <i>S. aureus</i> count (CFU/g)	
			1×10^2 – 1×10^4	1×10^5 – 1×10^6
Sheep cheese	100	60 (60%)	35 (35%)	25 (25%)
Dairy desserts	50	26 (52%)	14 (28%)	12 (24%)
Total	150	86 (57.3%)	49 (32.6%)	37 (24.6%)

2.8. Multiplex PCR (mPCR)

In this study, the primers and PCR assay conditions previously described by Sharma et al. (2000) were used for the simultaneous identification of the *sea*, *seb*, *sec* and *sed* gene sequences. PCR reaction was performed in a reaction mixture of 50 μ l final volume containing 2 μ l template DNA, 5 μ l $10 \times$ PCR buffer (Vivantis), 1 U Taq polymerase (Vivantis), 0.2 mM dNTP Mix (Vivantis), 4 mM $MgCl_2$ (Vivantis) and 20 to 30 pmol each of primers SA-U, SA-A, SA-B, SA-C/ENT-C, SA-D (Vivantis). The PCR amplification was performed with an initial denaturation of 94 °C for 5 min, followed by 35 cycles, each consisting of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s. Final extension cycle of 2 min at 72 °C (Techne TC-512). Amplification products were detected by agarose gel (1.5%) electrophoresis performed at 100 V for 40 min (EC250-90, Thermo, USA). The gels were stained with 0.5 μ l/ml ethidium bromide and inspected visually under a UV transilluminator (Vilber Lourmat, Marne La Vallee, France).

2.9. Enzyme-Linked Immunosorbent Assay (ELISA)

SEs were determined by using ELISA technique (Thermo, Finland) with commercially available kits (Ridascreen® SET A,B,C,D,E, r-biopharm, Germany, Art.no:R1101).

3. Results

CPS were found in 86 (57.3%) out of 150 analyzed samples. *S. aureus* were isolated from 60 (40%), 26 (17.3%) of sheep cheese and from of dairy desserts, respectively (Table 1). Total *S. aureus* counts determined on BPM (Oxoid) were between 1×10^2 and 1×10^6 CFU/g in sheep cheese and dairy dessert samples. In 25 (25%) of sheep cheese and 12 (24%) dairy dessert samples, CPS counts were above 10^5 CFU/g (Table 1). A total of 430 isolates from the 86 positive samples, 300 originated from cheeses and 130 dairy desserts were obtained in this study. 80 (18.6%) strains were characterized as *S. aureus*, being 60 (20%) from 300 cheese and 20 (15.3%) from 130 dairy desserts (Table 2). CPS isolates were analyzed by mPCR in order to detect *sea*, *seb*, *sec* and *sed* genes.

The ability to synthesize enterotoxins was determined in 12 (2.8%) out of 80 isolates by using ELISA technique. It was determined that these SEs had a distribution of 7 (1.6%) SEA, 2 (0.46%) SEB, 1 (0.23%) SEC, and 2 (0.46%) SED. Type A enterotoxin was found in 4 (1.3%) cheese and 3 (2.3%) dairy dessert samples. Type B enterotoxin was determined in 2 (0.6%) cheese samples. Type C enterotoxin was detected in only 1 (0.76%) dairy dessert. Type D enterotoxin was found in 1 (0.3%) cheese and 1 (0.76%) dairy dessert sample (Table 2). SEs genes were found in 13 (3.02%) of the total 80 isolates by using mPCR technique. It was determined that these enterotoxins contained 8 (1.8%) *sea*, 2 (0.46%) *seb*, 1 (0.23%) *sec* and 2 (0.46%) *sed* gene distribution, respectively. From cheese isolates, *sea* was detected in 5 (1.6%) isolates, *seb* was detected in 2 (0.6%) isolates and *sed* was detected in 1 (0.3%) isolate. From dairy dessert isolates, *sea* was detected in 3 (2.3%) isolates, *sec* was detected in 1 (0.76%) isolates and *sed* was detected in 1 (0.76%). In detail, these toxins were found in 3 (2.3%), 1 (0.76%) and 1 (0.76%) of tavuk gogsu, keskul and kazandibi samples, respectively (Fig. 1, Table 2). No SEs were identified in sutlac

Table 2
Prevalence of SEs genes in *S. aureus* isolates from samples.

Samples	Number of analyzed isolates	Total of CPS isolates	Number of SEs	SEs				Number of SEs gene	Enterotoxin gene			
				SEA	SEB	SEC	SED		sea	seb	sec	sed
Total	430	80 (18.6%)	12 (2.8%)	7 (1.6%)	2 (0.46%)	1 (0.23%)	2 (0.46%)	13 (3.02%)	8 (1.8%)	2 (0.5%)	1 (0.23%)	2 (0.46%)
Sheep cheese	300	60 (20%)	7 (2.3%)	4 (1.3%)	2 (0.6%)	–	1 (0.3%)	8 (2.6%)	5 (1.6%)	2 (0.6%)	–	1 (0.3%)
Dairy desserts	130	20 (15.3%)	5 (3.8%)	3 (2.3%)	–	1 (0.76%)	1 (0.76%)	5 (3.8%)	3 (2.3%)	–	1 (0.76%)	1 (0.76%)
Tavuk gogosu	30	5 (16.6%)	3 (2.3%)	2 (1.5%)	–	1 (0.76%)	–	3 (2.3%)	2 (1.5%)	–	1 (0.76%)	–
Keskul	40	5 (12.5%)	1 (0.76%)	1 (0.76%)	–	–	–	1 (0.76%)	1 (0.76%)	–	–	–
Kazandibi	30	6 (20%)	1 (0.76%)	–	–	–	1 (0.76%)	1 (0.76%)	–	–	–	1 (0.76%)
Supangle	15	2 (13.3%)	–	–	–	–	–	–	–	–	–	–
Sutlac	15	2 (13.3%)	–	–	–	–	–	–	–	–	–	–

and supangle. The distribution of SEs and SEs genes detected in dairy desserts was summarized in Table 2.

4. Discussion

S. aureus was isolated from cows, humans, processing equipment, the environment, milk, cheese at various stages of production (Jorgensen et al., 2005; Holeckova et al., 2002). This study was focused on the detection of enterotoxigenic *S. aureus* in sheep cheese and dairy desserts. In this study, *S. aureus* count was determined above 10^4 CFU/g in 25 (25%) sheep cheese samples (Table 1). However, *S. aureus* count was above the maximum tolerable microbiological limit (10^2 CFU/g) for cheese according to the Turkish Food Codex (Anonymous, 2001). Similar results were reported by several authors (Tawfek et al., 1989; Bostan et al., 2006; Pelisser et al., 2009). In addition, Necidová et al. (2009) reported *S. aureus* count higher than 10^5 CFU/g is unsuitable for the production of cheese. Indeed, staphylococcal counts should reach approximately 10^6 CFU/g to produce enterotoxin (Pelisser et al., 2009; Necidová et al., 2009). In the present study, enterotoxin-producing capacity was determined in 7 (2.3%) out of 60 sheep cheese isolates by using ELISA technique. These SEs had a distribution of 4 (1.3%) SEA and 2 (0.6%) SEB and 1 (0.3%) SED. SEs genes were identified only in 8 (2.6%) of 300 sheep cheese isolates. We found mainly *sea*, *seb* and *sed* genes in sheep cheese samples (Fig. 1 and Table 2). Similar results were presented by Normanno et al. (2005) and Tkacikova et al. (2003). In contrast, the incidence of *S. aureus* and their SEs was reported relatively high by several authors (Fotta et al., 2000; Holeckova et al., 2002) when

compared with our results. This might be due to differences in the reservoir in the various countries or ecological origin of strains, the sensitivity of detection methods, detected genes and number of samples, and kinds of examined samples included in these studies.

In this study, we analyzed dairy desserts which are considered to be traditional products in Turkey. CPS were found above 10^5 CFU/g in 12 dairy dessert samples (Table 2). These rates are above the legally permitted level (Anonymous, 2001). Similarly, Alisarlı et al. (2003) detected CPS counts were found above 10^3 CFU/g in 5 pudding samples. The ability of synthesizing enterotoxins was determined in 5 (3.8%) out of 20 dairy dessert isolates by using ELISA technique. It was determined that these SEs had a distribution of 3 (2.3%) SEA and 1 (0.76%) SEC and 1 (0.76%) SED. We mainly found *se* (*sea*, *sec*, and *sed*) genes in 5 (3.8%) of dairy dessert isolates (Fig. 1 and Table 2). Our study is the first report on the detection of SEs genes from dairy desserts in Turkey. There is not enough literature to dairy desserts in Turkey. Only Alisarlı et al. (2003) detected SEA, SEC and SEA/B in 10 (10%) of 100 dairy desserts by using ELISA. Similarly, Adesiyun and Balbirsingh (1996) were detected all (% 100) of 40 black pudding samples positive for *S. aureus* and they reported SEs (SEA, SEB, SEC and SED) in 27 (33.8%) of 80 isolates.

In comparison with other studies data which report that *seb*, *sec* or *sed* is mostly involved in food (Fotta et al., 2000; Holeckova et al., 2002; Normanno et al., 2005; Adwan et al., 2005), the largest percentage of *sea* (1.6%) genes was found in our study. Several authors have also reported that enterotoxin genes *sea* and *sed* are the most common in staphylococci isolated from food (Atanassova et al., 2001; Aragon-Alegro et al., 2007; Nájera-Sánchez et al. 2003). We observed that the incidence of *S. aureus* and SEs in dairy dessert samples is lower than

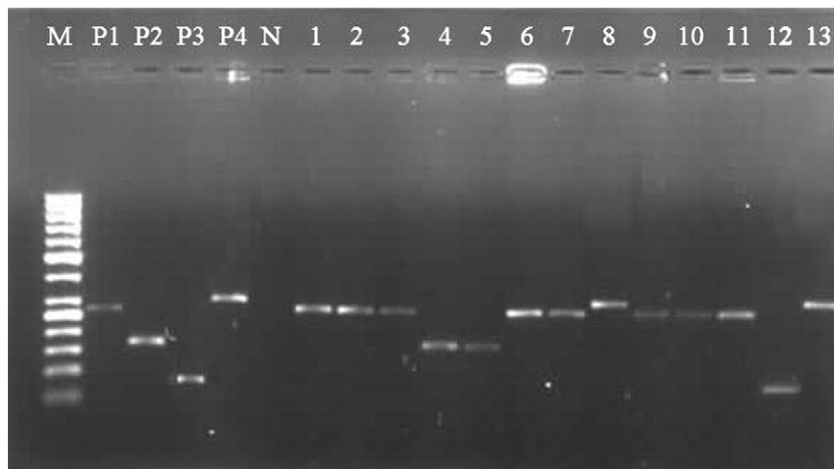


Fig. 1. Identification of *S. aureus* enterotoxin genes from sheep cheese and dairy dessert isolates by multiplex PCR. Lane M, molecular weight marker (Gene Ruler™ 50 bp DNA Ladder Plus, Fermentas); lane P1: positive control for *sea* (*S. aureus* ATCC 29231, 270 bp), lane P2: positive control for *seb* (*S. aureus* NCTC 10654, 165 bp), lane P3: positive control for *sec* (*S. aureus*, NCTC 10655, 69 bp), lane P4: positive control for *sed* (*S. aureus* NCTC 10652, 306 bp), lane N: Negative control (H₂O), lane 1–3: sheep cheese isolates, *sea*. Lane 4–5: sheep cheese isolates, *seb*. Lane 6–7: sheep cheese isolates, *sea*. Lane 8: sheep cheese isolates, *sed*. Lane 9–11: dairy dessert isolates, *sea*, lane 12: dairy dessert isolates, *sec*, lane 13: dairy dessert isolates, *sed*.

sheep cheese (Table 2). This might be due to the fact that cheese is mostly made from raw milk in Turkey and contributes to the sources of staphylococcal enterotoxigenesis. Moreover, Jørgensen et al. (2005) suggest that the bacteria were spread with the milk and product material to the equipment and the environment during milking and cheese production.

5. Conclusion

We detected that *S. aureus* which isolated from sheep cheese and dairy dessert samples have a heterogeneous enterotoxigenic potential. The results of this study suggested sheep cheese and dairy desserts had a potential health risk with regard to staphylococcal enterotoxins. The presence of *S. aureus* and SEs is an issue requiring consideration as it is relevant to food hygiene, especially regarding products derived from raw milk such as sheep cheese. Thus, raw milk should be pasteurized before cheese production. In order to reduce this risk factor to minimum, better hygiene practices are required in the production of the foods.

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