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Presence of *Staphylococcus aureus,* staphylococcal enterotoxins and antimicrobial resistance in traditionally produced raw milk cheeses

Vorhandensein von Staphylococcus aureus, Staphylokokkken Enterotoxinen und antimikrobielle Resistenzen in traditionell hergestelltem Rohmilchkäse

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Summary

The objectives of this study was to investigate the presence of *Staphylococcus aureus,* distribution of classical staphylococcal enterotoxin (SE) SEA to SEE, relevant gene/s and antimicrobial resistance pattern of *S. aureus* isolated from traditionally produced raw milk cheeses. A total of 106 fresh white cheese samples were examined. The 25 (23.6 %) of 106 cheese samples were found to be contaminated with coagulase positive staphylococci (CPS). From 52 isolates identified as *S. aureus,* one or more SEs was detected in 38.4 % of the isolates by ELISA whereas one or more *se* genes were detected in 50 % of the isolates by RT PCR. SEE (75 %) and *see* gene (61.5 %) were detected most frequently, whereas SED and *sed* gene were not detected in any isolates. Overall, 63.5 % of isolates were resistant to antimicrobial agents with 59.6 %, 13.5 %, 5.8 %, 5.8 % and 3.8 % of the isolates were resistant to penicillin, erythromycin, tetracycline, cefoxitin and kanamycin, respectively. The results of this study have revealed that cheeses made from raw milk were highly contaminated with *S. aureus,* therefore, creates a risk for public health due to the presence of enterotoxins as well as resistant strains against antimicrobial agents.

Keywords: *S. aureus,* staphylococcal enterotoxins, enterotoxin genes, antimicrobial resistance, raw milk cheese

Zusammenfassung

Ziel dieser Studie war die Untersuchung von Isolaten aus traditionell hergestelltem Rohmilchkäse auf die Präsenz von *Staphylococcus aureus,* das Vorhandensein der klassischen Enterotoxin (SE) Typen SEA-SEE sowie relevanter Gene und Resistenzmuster von *S. aureus* gegen antimikrobielle Varianten von *S. aureus.* Insgesamt wurden 106 weiße Frischkäse-Proben untersucht. 25 (23.6 %) der 106 Käseproben waren mit koagulasepositive Staphylokokken (CPS) kontaminiert. Bei 52 der Isolate wurde *S. aureus* identifiziert, durch ELISA wurden ein oder mehr SEs in 38.4 % der Isolate festgestellt, während ein oder mehr SEE-Gene in 50 % der Isolaten mittels RT-PCR nachgewiesen wurde. SEE (75 %) und SEE-Gen (61,5 %) wurden am häufigsten nachgewiesen, während SED und SED-Gene in keinem Isolat gefunden wurden. Insgesamt waren 63,5 % der Isolate resistent gegenüber antimikrobiellen Wirkstoffen. Resistent gegenüber Penicillin, Erythromycin, Tetracyclin, Cefoxitin und Kanamycin erwiesen sich 59,6 %, 13,5 %, 5,8 %, 5,8 % und 3,8 % der Isolate. Die Ergebnisse dieser Studie haben gezeigt, dass Rohmilchkäse stark mit *S. aureus* kontaminiert ist. *S. aureus* stellt aufgrund von Enterotoxinen und antimikrobiell resistenter Stämme ein Risiko für die öffentliche Gesundheit dar.

Schlüsselwörter: *S. aureus,* Staphylokokkken Enterotoxine, Enterotoxingene, Resistenzen, Rohmilchkäse

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Introduction

In recent years, there has been an increase in the number of consumers who prefer raw milk and dairy products, which are thought to be more natural and nutritious. The use of raw milk in cheese production generally increases the risk of staphylococcal food poisoning (SFP) due to contamination of products with *S. aureus* (Jørgensen et al., 2005; Akineden et al., 2008; Rosengren et al., 2010; Pedonese et al., 2014). Staphylococcal food poisoning caused by consuming contaminated foods with coagulase positive enterotoxigenic strains of Staphylococci (CPS) and staphylococcal enterotoxins (SEs) produced by particularly *S. aureus* is one of the most common foodborne disease in the world (Argudin et al., 2010; Ostyn, et al., 2012).

The growth of *S. aureus* and toxin production in milk and dairy products can vary depending on a large number of factors such as the health status of the animal (e. g. mastitis), inadequate hygienic practices during production, lack of pasteurization process and presence of competing microbia in the environment (Argudin et al., 2010; Pedonese et al., 2014; Poutrel et al., 2015; Mehli et al., 2017). Although *S. aureus* can be readily eliminated from food by pasteurization, the significance of SEs in terms of food safety is attributed to their feature of being heat resistant and their ability to maintain sufficient activity to cause intoxication after heat applications (Asao et al., 2003; Le Loir et al., 2003). *S. aureus* produce toxins as SEs and staphylococci-like enterotoxins (SE*l*). SEs and SE*l*s are traditionally classified as classical (SEA to SEE) and new (SEG to SE*l*/U2) types. It has been reported that, in general, 95 % of staphylococcal food intoxications are due to SEA-SEE types and the remaining 5 % intoxication may be due to newly identified SEs (Argudin et al., 2010). The most common enterotoxin in staphylococcal food poisonings is SEA, which is followed by SED and SEB (Argudin et al., 2010; Rosengren et al., 2010). Although the food consumption patterns associated with SFP may vary from country to country according to consumption habits, SFP cases associated with milk and dairy products, especially consumption of cheese produced from raw milk, are frequently reported (Asao et al., 2003; Ostyn et al., 2010; Johler et al., 2015; Poutrel et al., 2015).

Antimicrobial resistance is also an important issue for public health all over the world. The widespread and unconscious use of antibiotics in the agricultural and livestock sectors leads to the development of many resistant bacteria. *S. aureus* is an important pathogen due to antibiotic resistance (Le Loir et al., 2003). Recently it has been reported that there is an increase in the development of resistance to antimicrobials in *S. aureus* strains as well as in other pathogenic agents isolated from food (Normanno et al., 2007; Masalankova, et al., 2009; Can and Çelik, 2012).

Along with the cheese production in industrial milking enterprises by modern technological methods, small scale farms in the rural areas of Southeastern part of Turkey continue to produce raw milk cheeses as in other parts of the country. Some of the cheeses are consumed by households and the remaining cheeses are sold in bazars and small retail shops. The aim of this study was to determine the frequency of *S. aureus,* presence of staphylococcal enterotoxins and enterotoxin genes in *S. aureus* isolates and the susceptibility of isolates to antimicrobial agents in cheeses, which are produced by a traditional way from raw milk to evaluate the possible risk situation in terms of public health.

Materials and methods

Collection of traditional fresh raw milk cheese samples

Mardin is located at 37°18′46″N and 40°44′2″E and almost all of the traditionally produced raw milk cheese made by mixing sheep and goat milk. A total of 106 fresh white cheese samples were purchased from retail shops in Mardin city center. After 1–2 days of the production, approximately 5–10 kg of cheeses in buckets, which have the owners' name on it, are brought by the farmers to retail shops for sale. Thus, in this study, all of the samples were taken from cheeses produced from raw milk in different small farms. From each bucket from different retail shops 100 g of cheese samples were collected into sterile plastic bags between April and May 2016. The samples were transported to the laboratory in a refrigerated box (Approx. 4 °C) aseptically in cold chain and stored 2–8 °C until analysis. The analyses were performed as soon as possible.

Isolation and identification of *S. aureus*

For isolation and identification of *S. aureus,* a 10 g portion of each sample was put into a sterile plastic bag and 90 ml of peptone water (Merck, M.07228.0500) was added and samples were homogenized for 2 minutes on a stomacher (Smasher Lab Blender). The homogenized samples were inoculated onto Baird Parker Agar (Oxoid, CM0275) supplemented with 5 % egg yolk and tellurite (Merck, Darmstadt, Germany) and incubated at 37 °C for 24 to 48 h. Colonies with a typical black appearance and surrounded by clear zone were evaluated as CPS. One-five selected typical colonies were tested for *S. aureus* using Dry Spot Staphytect Plus Test (Oxoid, DR0100M). The 52 *S. aureus* positive isolates were used to detect staphylococcal enterotoxins, enterotoxin genes and antimicrobial susceptibilities.

Detection of staphylococcal enterotoxins (SEs) by ELISA

The *S. aureus* strains, grown in tubes containing 10 ml of Brain Heart Infusion Broth (Oxoid CM0225) at 37 °C for 24 h, were centrifuged at 3500 x g for 5 min, and the supernatants were applied through a filtering membrane system with pore diameters of 0.2 mm (Minisart, Sartorius Stedim Biotech GmbH, Oettingen, Germany) for sterilization. A volume of 100 µl of the filtrate per well was used in the enzyme-linked immunosorbent assay (ELISA). The samples were tested for SEA, SEB, SEC, SED and SEE enterotoxins using commercial ELISA RIDASCREEN kit (R-Biopharm AG, Darmstadt, Germany. Art. No: R4101) according to the manufacturer's instructions.

Detection of staphylococcal enterotoxin genes by RT-PCR

Automated DNA extraction

DNA extraction was performed from bacterial colonies and High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany) was used according to manufacturer instructions to obtain pure bacterial DNA (Reischl et al., 2000). DNA concentration and purity were measured for all isolations using ND-1000 spectrophotometer (NanoDrop Technologies, USA). Each measurement was repeated three times. DNA from each isolates was stored at –20 °C until analysis by RT PCR.

Primers and probes used for detection of staphylococcal enterotoxin genes

Specific primers and probes labeled with 6-carboxy-fluorescein (FAM) at 5' end and with carboxymethylrhodamine (TAMRA) at the 3' end were used to detect *S. aureus sea, seb, sec1, sed* and *see* genes. Primers and probes were supplied by IDT (Integrated DNA Technologies, Inc., Coralville, IA). The primers and probes used in this study were presented in Table 1.

Real-time PCR procedures were performed according to the instructions of manufacturer using the LightCycler 480 Probe master kit (Roche Diagnostics GmBH, Mannheim, Germany) in a LightCycler 480 II (Roche Diagnostics GmBH, Mannheim, Germany) system. 0.1 µMol probe and 0.5 µMol primers were added to the each reaction in final concentration and a 5 µl of template DNA was used in 20 µl total reaction mixture.

The PCR amplification was performed with an initial denaturation at 94 °C for 10 min which was followed by 40 cycles, each consisting of 95 °C for 10 s, 55 °C for 30 s and 72 °C for 1s. Finally 72 °C for 2 min for a final extension was performed. Fluorescence signals were collected by a single read acquisition at 72 °C.

Antimicrobial susceptibility testing

All of the 52 *S. aureus* isolates were tested by disk diffusion method on Mueller Hinton Agar for the detection of antimicrobial susceptibilities and the results were evaluated according to CLSI 2015 (CLSI, 2015). Antimicrobial Susceptibility Test Discs (Oxoid, Basingstoke, UK) containing 2 µg clindamycin, 30 µg teicoplanin, 15 µg erythromycin, 10 µg gentamicin, 30 µg kanamycin, 30 µg cefoxitin, 10U penicillin G, 30 µg tetracycline were used. *S. aureus* ATCC 25923 was used as a reference strain.

Results and Discussion

According to Turkish Legislation, the number of CPS should not be more than 10^3 cfu/g in all cheeses, except melting cheeses (Turkish Food Codex, 2011). In this study, it was determined that 25 (23.6 %) of the 106 cheeses produced from raw milk by traditional methods were found to be contaminated with *S. aureus.* This contamination rate was very closely compatible with the findings of Koluman et al. (2011) who found 21.3 % contamination rate. Whereas lower than the contamination rates of 60 % Ertas et al. (2010) and 36 % Tasci et al. (2011) determined on different types of cheese in other regions of Turkey. On the other hand, the contamination rate in the present study was higher than the rate (6%) determined by Can and Çelik (2012).

In studies conducted in other countries, the contamination rates were determined as 7.7 % by Akineden et al. (2008) in Germany, as 69 % by Rosengren et al. (2010) in Sweden, as 10.9 % by Jamali et al. (2015) in Iran, as 33.9 % by Ferreira et al. (2016) in Brazil and as 69.2 % by Rola et al. (2016) in Poland. However, Belickova et al. (2001) have reported the absence of *S. aureus* in cheese samples from Slovakia.

Cheese samples tested in this study were produced from raw milk by family members on the farm. Rosengren et al. (2010) reported that more than one *S. aureus* strains were detected in the same cheese sample produced on the same farm indicating that contamination of the cheese with *S. aureus* may result from more than one factor. In the present study, it is thought that the production of the cheese from raw milk is one of the most important factors responsible for the contamination of 23.6 % of the cheese with *S. aureus.* It has been stated that the CPS rate in the cheeses produced from raw milk is 69 % whereas 6 % in the cheeses made from pasteurized milk (Rosengren et al., 2010). The health of the milking animals and the cheese makers, the equipment used and the environmental contamination are also possible factors for the determined contamination rate in the present study. In a study conducted by Hummerjohann et al. (2014) *S. aureus* strains associated with mastitis were isolated from cheese contaminated with CPS. Mehli et al. (2017) and Rola et al. (2016) reported that equipment used in cheese production and personnel were contaminated with CPS.

Fifty-two *S. aureus* isolates were obtained from 25 positive samples for CPS. These isolates were tested for enterotoxin production by ELISA and for *se* genes by RT PCR. Staphylococcal enterotoxin and/or *se* gene (s) were detected in 26 isolates (50 %) obtained from 15 independent cheese samples. One or more SEs were detected in 20 (38.4 %) by ELISA whereas one or more *se* genes were detected in 26 (50 %) isolates by RT PCR (Table 2). In this study, positivity rate for SE/s production by ELISA was rather higher than the positivity rates determined by Tasci et al. (2011) and Can and Çelik (2012). Moreover, detection rate of *se* gene/s by RT PCR was higher than positivity rate of 2.6 % determined by Ertas et al. (2010).

TABLE 1: *The sequences of oligonucleotide primers and probes used in RT PCR*

Gene	Primer & Probe	Sequence	Amplicon Size	Genbank	References
SEA	SEA fw SEA Rv SEA Probe	5-AAAATACAGTACCTTTGGAAACGGTT-3 5-TTTCCTGTAAATAACGTCTTGCTTGA-3 FAM-AACGAATAAGAAAAATGTAACTGTTCAGGAGTTGGATC-Tamra	92	M18970	(Klotz et al,, 2003)
SEB	SEB fw SEB Rv SEB Probe	5-ACACCCAACGTTTTAGCAGAGAG-3 5-CCATCAAACCAGTGAATTTACTCG-3 FAM-CAACCAGATCCTAAACCAGATGAGTTGCACA-Tamra	81	M11118	(Klotz et al., 2003)
SEC	SEC fw SEC Rv SEC Probe	5-AATAAAACGGTTGATTCTAAAAGTGTGAA-3 5-ATCAAAATCGGATTAACATTATCCATTC-3 FAM-TAGAAGTCCACCTTACAACAA-Tamra	80	X05815	(Klotz et al., 2003)
SED	SED fw SED Rv SED Probe	5-TGATTCTTCTGATGGGTCTAAAGTCTC-3 5-GAAGGTGCTCTGTGGATAATGTTTT-3 FAM-TATGATTTATTTGATGTTAAGGGTGATTTTCCCGAA-Tamra	115	M28521	(Klotz et al., 2003)
SEE	SEE fw SEE Rv SEE Probe	5-GCTTTGGCGGTAAGGTGC-3 5-ATAACTTACCGTGGACCCTTCAGA-3 FAM-AGGCTTGATTGTGTTTCATT-Tamra	68	M21319	(Chiefari et al., 2015)

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TABLE 2: *SE (A to E) and se (sea to see) gene profiles of S. aureus isolates*

Sample no	SE by ELISA					*se* genes by RT PCR				
	SEA	SEB	SEC	SED	SEE	*sea*	*seb*	*sec*	*sed*	*see*
3							x			x
8					x					x
13-2					x					x
30-2							x			x
30-3					x					x
36-1					x					x
36-2					x					x
43-1	x	x	x			x	x	x		
43-2							x			
43-3	x	x	x			x	x	x		
66-1					x					x
66-2	x	x	x		x	x	x	x		nd
66-3					x					x
76-2					x		x			x
80-2							x			
80-3							x			
81-1							x			
89-1					x			x		x
89-2					x					x
98-2					x					x
99-1					x			x		x
99-3					x					x
100-1	x	x	x			x	x	x		
100-2		x	x				x	nd		
100-3	x	x	x			x	x	x		
105-1					x		x			x
Total	**5**	**6**	**6**	**0**	**15**	**5**	**14**	**7**	**0**	**16**

nd: not determined

The results of both tests were found to be 53.8 % compatible for SEs production and the presence of *se* genes, which is lower than the 80 % compliance rate determined by Ostyn et al. (2012) for quantitative ELISA confirmatory method and PCR test results. Ten (38.4 %) of the isolates exhibited the genes that were *seb, see* in 2, *seb* in 6 and *sec* in 2 isolates by RT PCR but SEs that are the product of these genes were not detected by ELISA (Table 2). Similar to our study, previous studies have also shown that a higher number of se genes having enterotoxin production potentials were detected by PCR compare to immunologic test methods (Loncarevic et al., 2005; Ostyn et al., 2012). These results can be interpreted as the fact that although the genes that can produce SEs are present in the genome of *S. aureus,* either they are not transcribed for SEs production or their products are below the detection level of immunologic tests like ELISA. On the other hand, these genes representing SEE and SEC detected by ELISA were not detected by RT PCR in 2 (7.6 %) isolates (Table 2). This result confirms the results of Ostyn et al. (2012) and Pereira et al. (2009) that may be a false positivity resulting from nonspecific SE reactions due to milk derived endogenous enzymes existing in the cheese (Ostyn et al., 2012).

In this study SEE was detected in 15 (75 %) of the 20 positive isolates by ELISA; SEB was detected in 6 (30 %) isolates, SEC was detected in 6 isolates (30 %), which is followed by SEA in 5 (25 %) isolates, whereas *see* genes were detected in 16 isolates (61.5 %) out of 26 isolates by RT PCR; *seb* was detected in 14 (53.8 %), *sec* was detected in 7 (26.9 %) and *sea* was detected in 5 (19.2 %) isolates (Table 3). In consistent with the results of Akineden et al. (2008) and Rosengen et al. (2010), SED and sed were not detected either by ELISA or by RT-PCR in this study.

It has been indicated that SEA is frequently produced by *S. aureus* strains isolated from human, SEC or SED are produced by the strains isolated from cattle and SEC produced by strains isolated from sheep (European Commission, 2003). Ostyn et al. (2012) have reported that *sed* gene is determined in 21 (81%) of 26 isolates from cow milk-derived cheeses. The distribution of SE types may be dependent on the sources of contamination. In the present study, the absence of SED and *sed* in the tested samples may be due to the fact that cheese production in Mardin city is mostly done by mixing sheep and goat milk. Ostyn et al. (2010) reported that SEE was identified in cheese produced from raw milk in six food poisoning cases in France in 2009. Tasci et al. (2011) reported that they detected SEE only in one cheese sample. Rola et al. (2016) reported that the *se* genes they have identified in their studies are different from the studies done in some other countries, which may be due to the presence of different strains of *S. aureus* in different geographical regions. Poli et al. (2007) indicated that there were seasonal changes in the presence of *se* gene types detected. The reasons for the frequent detection of SEE and *see* genes at higher rate in this area and the determination of contamination sources need further investigations.

In the present study, 33 (63.5 %) of 52 isolates were found to be resistant to one or more antimicrobial agents (Table 4), which was lower than the results of studies previously conducted in Turkey by Koluman et al. (2011) and Can and Çelik (2012) whereas slightly higher than the resistance rate

TABLE 3: *The correlation between the SE production and the presence of enterotoxin genes*

Type of SEs	Number of SE by ELISA, %	Number of se genes by RT-PCR, %
A, B, C, E	1 (5)	–
A, B, C	4 (20)	5 (19.2)
B, C	1 (5)	–
B, E	–	4 (15.4)
C, E	–	2 (7.7)
B	–	5 (19.2)
E	14 (70)	10 (38.5)
Total	**20**	**26**

TABLE 4: *Antimicrobial sensitivity of S. aureus isolated from cheese samples and resistance profiles of the isolates*

Antimicrobial Agents	Disc Content	Antimicrobial sensitivity of isolates n:52			Resistance profile of isolates n:33		
		Susceptible n (%)	Intermediate n (%)	Resistant n (%)	Single n:28	Double n:2	Multidrug n:3
Clindamycin	2 µg	52 (100)	0	0	–	–	–
Teicoplanin	30 µg	49 (94.2)	3 (5.8)	0	–	–	–
Erythromycin	15 µg	42 (80.8)	3 (5.8)	7 (13.5)	2	2	3
Gentamicin	10 µg	52 (100)	0	0	–	–	–
Kanamycin	30 µg	50 (96.2)	0	2 (3.8)	–	–	2
Cefoxitin	30 µg	49 (94.2)	0	3 (5.8)	–	–	3
Penicillin G	10U	21 (40.4)	–	31 (59.6)	26	2	3
Tetracyline	30 µg	49 (94.2)	0	3 (5.8)	–	–	3

found by Ferreira et al. (2016). However, Mehli et al. (2017) found that all isolates they tested were susceptible antibacterial agents, in this study 16 (30.7 %) isolates were susceptible to all tested antimicrobial agents.

Twenty-eight (84.8 %) of the 33 resistant isolates were resistant to a single antimicrobial agent. Twenty-six isolates were resistant to only penicillin and 2 isolates were resistant to only erythromycin. Two isolates (6.0 %) were resistant double antimicrobial agents (penicillin and erythromycin). The remaining 3 (9.3 %) isolates, were multidrug resistant. These isolates (isolate no: 66/1, 66/2, 66/3) obtained from one sample were resistant to erythromycin, penicillin tetracycline and cefoxitin. In addition, the isolates 66/1 and 66/2 were also resistant to kanamycin. The 3 cefoxitin resistant isolates (5.8 %, 3/52) were considered as oxacillin and methicillin resistant according to the cefoxitin results (Table 4). All isolates were susceptible to clindamycin and gentamicin.

The 31 (59.6%) of the *S. aureus* isolates were identified as penicillin resistant, which was the highest rate among the tested agents. Similarly, Ferreira et al. (2016) found the 44.8 % of the isolates resistant to penicillin. Jamali et al. (2015) have also found that 47.3 % of the isolates from the milk and milk products and 51.1 % of the isolates from traditionally produced cheese were resistant to penicillin. Rola et al. (2016) reported the resistance rate to penicillin as high as 50.8 %. In the present study, following penicillin, 7 (13.5 %) of the isolates were erythromycin, 3 (5.8 %) were tetracycline, 3 (5.8 %) were cefoxitin and 2 (3.8 %) were kanamycin resistant. Jamali et al. (2015) found that the rate of tetracycline resistance in traditional cheeses was 51.1 %, which was quite higher than our results, while the 8.2 % of the resistance rate against cefoxitin (5.8 %) was similar to our findings. Although, no clindamycin resistant isolates were detected in our study, Jamali et al. (2015) determined 14.3 % resistance rate against clindamycin. The high resistance rates to antimicrobial agents determined in the present study can be attributed to the fact that these agents are used in control and treatment of animal disease without regarding the frequency and usage conditions of these agents.

Conclusion

The results of this study have shown that cheese made from raw milk is highly contaminated with *S. aureus*. In addition, this study provided the information about the distribution rate of SE types and *se* genes in these isolates. Since, the presence of *S. aureus* in cheese poses a risk for public health due to both the presence of enterotoxins and resistant strains against antimicrobial agents, it is necessary to establish an effective control system and to increase the awareness of producers to ensure safety of the production.

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Conflict of interest

The authors have no conflict of interest to declare.

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