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RESEARCH ARTICLE

Staphylococcal Enterotoxins and Enterotoxigenic *Staphylococcus aureus* in Raw Milk: A Screening Study

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ABSTRACT

Staphylococcus aureus is one of the most important cause of foodborne intoxications in human beings. Staphylococcal enterotoxins (SEs) may lead to outbreaks because of taking food such as milk and dairy products. The aims of this study were to analyze the presence of staphylococcal enterotoxins and enterotoxigenic properties of the *S. aureus* isolates in 120 raw milk samples. One hundred and twenty raw milk samples were analyzed to detect SEs using the enzim-linked immunosorbent assay (ELISA) method. Staphylococcal entertoxin genes (*sea, seb, sec, sed, see*) were analysed by polymerase chain reaction (PCR). In the current study, SEs were found 2 of 120 bulk tank milk samples. Totally 18 (38.3%) of 69 isolates were confirmed by PCR targeting *nuc* and *coa* genes in *S. aureus*. SEs genes were detected as 3 (16.6 %) of 18 *S. aureus* isolates. Staphylococcal enterotoxins in foods like milk and dairy products are the potential public health hazards. Surveillance programs and effective monitoring systems are required for controlling staphylococcal enterotoxins in raw milk.

Keywords: Raw milk, Staphylococcal enterotoxins, *Staphylococcus aureus*

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Çiğ Sütte Stafilokokal Enterotoksinler ve Enterotoksijenik *Staphylococcus aureus* Varlığının Belirlenmesine Yönelik Bir Tarama Çalışması

ÖZ

Staphylococcus aureus, insanlarda gıda kaynaklı zehirlenmelerin başlıca nedenidir. Stafilokokal enterotoksinler (SE'ler) ile kontamine süt ve süt ürünleri tüketimi salgınlara neden olabilmektedir. Bu çalışmanın amacı, 120 çiğ süt örneğinde *S. aureus* izolatlarının stafilokokal enterotoksinlerin ve enterotoksijenik özelliklerinin analiz edilmesidir. SE'leri saptamak için enzim bağlantılı immünosorban testi (ELISA) yöntemi kullanılarak yüz yirmi çiğ süt örneği analiz edildi. Polimeraz zincir reaksiyonu (PCR) ile stafilokokkal entertoksin genleri (*sea*, *seb*, *sec*, *sed*, *see*) araştırıldı. Bu çalışmada, toplama tanklarından alınan 120 çiğ süt örneğinden 2'sinde SE tespit edilmiştir. Toplam 69 *S. aureus* izolatının 18'i (% 38.3) *nuc* ve *coa* genleri PCR yöntemi ile doğrulanmıştır. SE genleri, 18 *S. aureus* izolatının 3'ünde (%16,6) bulunmuştur. Süt ve süt ürünlerinde bulunan stafilokokal enterotoksinler halk sağlığı açısından potansiyel tehlikedir. Çiğ sütteki stafilokokal enterotoksinlerin kontrolü için sürveyans programları ve etkili izleme sistemleri gereklidir.

Anahtar Kelimeler: Çiğ süt, Stafilokokal enterotoksinler, *Staphylococcus aureus*

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INTRODUCTION

Milk and dairy products are a great protein source especially for children in the age of growth (Kandpal et al. 2012). Milk is also suitable medium for foodborne pathogens which can cause a major public health risk (Ding et al. 2016). Although milk is sterile during secretion, it can be contaminated by microorganisms during milk handling, storage and processing. (De Silva et al. 2016). Foodborne outbreaks caused by milk and dairy products have led to hospitalizations and deaths for human beings (Painter et al. 2013).

Staphylococcus aureus is recognized worldwide as a major foodborne pathogen and it has to produce a varitey of toxins which cause staphylococcal food poisoning (SFP) in human (Le Loir et al. 2003, Ote et al. 2011). *S. aureus* is normally found in healthy nose and skin mucosa in human (Kluytmans et al. 1997). Also, the presence of biofilm producing ability of *S. aureus* in milk and milking environment is a public health concern for the consumers (Lee et al. 2014, Lee et al. 2016). *S. aureus* produces a variety of toxins called staphylococcal enterotoxins (SE) (Kuzma et al. 2003, Ozdemir and Keyvan 2016). Staphylococcal enterotoxins are divided as classical and new SE like toxins (SEls). Current studies have described 23 SEs and SEls (Benkerroum 2018). Not only SE, but also SE/SEls can lead to staphylococcal foodborne outbreak (Umeda et al. 2017). The presence of a small amount of staphylococcal enterotoxins can cause an intoxication that results from the consumption of contaminated food (Berdgoll 1989). SFP is generally self limiting and symptoms of are abdominal cramp, nausea, vomiting and with or without diarrhea (Argudín et al. 2010). Consumption of contaminated milk and dairy products are the main source of enterotoxins for human (Normanno et al. 2007, Lee et al. 2012). SEs led to outbreak because of the consumption of contaminated milk and dairy products (Schmid et al. 2009, Umeda et al. 2017)

S. aureus is also causative agent of mastitis in dairy cows (Peles et al. 2007). Dairy products may create a human illness due to contamination of milk with *S. aureus* (Jørgensen et al. 2005a, Duquenne et al. 2010). Subclinical mastitis, improper milking conditions during milking in dairy cows are the possible contamination ways of raw milk with *S. aureus* (Jørgensen et al. 2005b). Pasteurization process can inactivate *S. aureus* but thermostable SEs may retain biological activity (Schmid et al. 2009). Thus, detection of the staphylococcal enteretoxins and enterotoxic strains in foods is required (Morandi et al. 2007). The objective of this study were to detect stapylococcal enterotoxins and related genes in *S. aureus* isolated from bulk tank milk samples.

MATERIALS and METHODS

Milk samples

In this study, a total of 120 raw milk samples were obtained in Burdur province, located in the southern side of Turkey. Fifty ml of each milk sample was taken in sterile plastic collection tubes and transported to the laboratory under refrigeration (4°C–8°C), and the samples were directly processed for further analyses.

Isolation and identification of *S. aureus*

Serial dilutions were prepared homogenously in aseptic conditions from milk samples and inoculated on Baird Parker / RPF (BP + RPF Oxoid, CM0961) agar (Bennett and Lancette 2001). Milk samples were incubated at 35ºC for 24-48 hours. Then, typical and atypical colonies were selected, and coagulase test was performed with EDTA coagulase plasma (Oxoid, R21052). Coagulase test positive colonies were analysed for Gram staining, catalase test, DNase activity, hemolytic properties (ß-hemolysis) and mannitol fermentation test. Phenotypically positive colonies from these tests were accepted as suspected isolates of *S. aureus* (ISO 2003, Parisi et al. 2016).

DNA isolation

Overnight cultures in Brain Heart Infusion broth (BHI, Oxoid, CM1135) were used for the DNA isolation. For this purpose, 2 ml of broth cultures were centrifuged at 5.000 g. 10 minutes and the supernatant were discarded. Bacterial pellets were washed twice with 1 ml of saline solution and centrifuged again. Bacterial pellets were resuspended in 180 µl Tris EDTA buffer (Sigma-Aldrich, 93283) containing 18 µl of lysostaphin (0.5 U/µl, Sigma, L7386) and incubated at 37°C for 1 hour (Akinedan et al. 2008). Genomic DNA was extracted according to GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA) manufacturer's protocol. A nano-drop (NanoDrop2000-Thermoscientific™) technique was used to define the quantification of DNA.

PCR analyses

In the current study, *S. aureus* ATCC 25923, *S. aureus* NCTC 10652 FDA 196E (*SEA*), *S. aureus* NCTC 10654 FDA 243 (*SEB*), *S. aureus* NCTC 10655 137 (*SEC*), *S. aureus* NCTC 10656 494 (*SED*) strains used as positive control. *S. aureus* reference strain for *SEE* was kindly provided by Dr. Ömer Akineden (Dairy Sciences, Institute of Veterinary Food Sciences, JustusLiebig- University Giessen, Germany). *S. aureus* isolates were confirmed related *coa* and *nuc* gene primers showing in Table 1. Staphyloccoccal enterotoxin genes were detected by PCR method. For this purpose, *S. aureus* isolates were analysed for the presence of *sea*, *seb*, *sec*, *sed*, *see* genes related primers showing in Table 1. In the current study, annealing temperatures of all genes were detected by gradient

PCR. Except for *nuc* and *coa* genes, all genes were analysed by uniplex PCR method. The PCR reaction mixture was prepared from 3 µl of DNA, 0,5 µl of each primer, 4 µl of 5x FIREPol® Master Mix (Solis Biodyne, Tartu, Estonia), 12 µl of water, for a total reaction volume of 20 µl. The ampification conditions were 95 °C for 4 min, followed by 30 cycles at 95 °C for 30 s, 55°C (*nuc*, *coa*, *seb*) to 56.5°C (*sea*, *sed*, *sec*, *see*) for 1 min s, and 72 °C for 40 s and a final extension step of 72°C for 10 min. The amplified PCR products were observed in 1.5% agarose gel electrophoresis (Keyvan and Ozdemir, 2016).

Detection of Staphylococcal Enterotoxins

Staphylocccal enterotoxins (SET A, B, C, D, E) in raw milk samples were analyzed according to Ridascreen® SET A,B,C,D,E (r-biopharm, Germany, Art.no:R1101) test kit procedure by Enzim-linked immunosorbent assay (ELISA) method. For this purpose, 10 ml of milk sample was centrifuged at 3500 g/10min/10°C and cream layer discarded. The supernatant was used for the detection of enterotoxins. The absorbance value of milk samples was obtained from the ELISA plate reader at 450 nm (ELX-800; Bio-Tek Instruments, Winooski, VT, USA).

Table 1. Primers used in this study
Tablo 1. Çalışma kapsamında kullanılan primer dizileri.

Target gene	Primer sequence (5' 3')	Product size (bp)	References
nuc	F: ATA GGG ATG GCT ATC AGT AAT GT R: GAC CTG AAT CAG CGT TGT CTT C	624 bp	Lem et al. (2001)
coa	F: GTA GAT TGG GCA ATT ACA TTT TGG AGG R: CGC ATC AGC TTT GTT ATC CCA TGT A	117 bp	Kearns et al. (1999)
sea	F: GGT TAT CAA TGT GCG GGT GG R: CGG CAC TTT TTT CTC TTC GG	102 bp	Mehrotra et al. (2000)
seb	F: GTA TGG TGG TGT AAC TGA GC R: CCA AAT AGT GAC GAG TTA GG	164 bp	Mehrotra et al. (2000)
sec	F: AGA TGA AGT AGT TGA TGT GTA TGG R: CAC ACT TTT AGA ATC AAC CG	451 bp	Mehrotra et al. (2000)
sed	F: CCA ATA ATA GGA GAA AAT AAA R: ATT GGT ATT TTT TTT CGT TC	278 bp	Mehrotra et al. (2000)
see	F: AGG TTT TTT CAC AGG TCA TCC R: CTT TTT TTT CTT CGG TCA ATC	209 bp	Mehrotra et al. (2000)

RESULTS

In the current study, 18 (38.3%) of 69 the *S. aureus* isolates were confirmed by PCR targeting *nuc* and *coa* genes in *S. aureus* (Fig. 1). In this study, classical enterotoxins were detected by Ridascreen and *S. aureus* isolates from bulk tank milk contained classical enterotoxins genes. *seb* and *sec* gene were found as 3 (16.6%) of 18 *S. aureus* isolates.

The Ridascreen® SET A, B, C, D, E test procedure indicates two assessment option for the detection of staphylococcal enterotoxins. First way is the visual determination of the color change after the addition of the stop solution and second way is the calculation of the cut off value. The cut off value is found by adding 0.15 to the negative control absorbance value. Results of the absorbance values are equal or above to the cut off value which are considered positive while results are below the cut off value are that samples considered as negative for staphylococcal enterotoxins.

Based on our results, according to the visual determination staphylococcal enterotoxins were detected as positive 4 of 120 bulk tank milk samples while 2 of 120 bulk tank milk samples were found as positive in assessment of the cut off value (Table 2 and Table 3).

DISCUSSION

Milk is a suitable medium for *S. aureus* growth and enterotoxin production. Pasteurization process can inactivate *S. aureus* from raw milk but SEs will remain stable even after heat treatment (Le Loir et al. 2003, Lee at al. 2012). Rall et al. (2008) was observed that the presence of enterotoxigenic *S. aureus* even after pasteurization. The reason for this, it could be the possible inefficacy of the thermal process.

SEs are the most prevalent agent of milk-borne intoxications causing risk on the public health worldwide (Benkerroum 2018). In the current study, staphylococcal enteretoxins were detected in 2 of 120 (1.66%) bulk tank milk samples. In a study from

Norway, enterotoxin production was identified 22.1% of *S. aureus* isolates in bovine milk tank and SE genes were found 52.5% of the isolates (Jørgensen et al. 2005a). Previous studies from different countries were reported levels of enterotoxigenic *S. aureus* as 9.4 %, 20 %, 37.1 %, 13.1%, 26.1%, 27.1% in Jordan, Portugal, Czech Republic, Poland, Egypt, Hungary, respectively (Peles et al. 2007, Zouharova and Rysanek 2008, Pereira et al. 2009, Mansour et al. 2017, Korpysa-Dzirba and Osek 2018, Obaidat et al. 2018). Enterotoxigenic *S. aureus* isolates in raw milk may pose potential public health hazard and due to thermostable enterotoxins, dairy products may cause intoxications in humans. Schmid et al. (2009) were reported an outbreak because of consumed school milk products in Austria.

Table 2. Absorbance value of bulk tank milk samples
Tablo 2. Süt toplama tank örnekleri absorbans değerleri.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	0.792	0.043	0.043	0.044	0.200	0.045	0.044	0.044	0.045	0.045	0.047	0.049
B	0.712	0.045	0.043	0.044	0.044	0.045	0.044	0.044	0.044	0.044	0.044	0.047
C	0.042	0.047	0.043	0.043	0.044	0.044	0.043	0.044	0.044	0.045	0.044	0.047
D	0.042	0.043	0.045	0.044	0.044	0.046	0.044	0.047	0.052	0.044	0.044	0.046
E	0.043	0.043	0.044	0.098	0.059	0.044	0.044	0.044	0.044	0.046	0.045	0.047
F	0.043	0.045	0.046	0.049	0.046	0.045	0.044	0.045	0.045	0.045	0.045	0.048
G	0.045	0.043	0.044	0.044	0.044	0.045	0.046	0.044	0.045	0.045	0.047	0.047
H	0.045	0.045	0.047	0.205	0.047	0.045	0.044	0.050	0.045	0.045	0.046	0.047

1A/1B: Positive Control, 1C/1D: Negative Control, Cut off value: 0.192, 4/H-5/A: Samples are above to cut off value

Table 3. Staphylococcal enterotoxins (A, B, C, D, E) in raw milk samples
Tablo 3. Çiğ süt örneklerinde stafilokokal enterotoksinler (A, B, C, D, E).

Number of samples	Color change Positive samples	Cut off value Positive samples
120	4 (3.3%)	2 (1.6%)

Table 4. Enterotoxigenic properties of *S. aureus* isolates
Tablo 4. *S. aureus* izolatlarının enterotoksijenik özellikleri.

Target gene	Number of positive *S. aureus* isolates (n=18)
sea	-
seb	2 (11.1%)
sec	-
sed	-
see	1 (5.5%)
Total	3 (16.6)

Mastitis is one of the most economically devastating problems in cattle and *S. aureus* is a common causative agent of clinical and subclinical mastitis (Türkyılmaz et al. 2010, Ote et al. 2011, Rall et al. 2014). In Brasil, *S. aureus* was isolated in 6.7% of raw milk samples from dairy cows with subclinical mastitis and 10.8% of bulk tank milk samples. Also, four of *S. aureus* isolates were reported enterotoxigenic. (Fagundes et al. 2010). Boynukara et al. (2008) was found to be enterotoxigenic 25.5% of *S. aureus* strains isolated from cows with subclinical mastitis. Rall et al. (2014) were observed that 53.3% of S. aureus isolates contained *sea* gene in milk from cows with subclinical mastitis. Milk collected from dairy cows with subclinical mastitis may pose a significant source of enterotoxigenic *S. aureus* which can produce SEs. Transfer of the contaminated milk to bulk tank milk may cause intoxitacations. Ding et al. (2016) were recommended that to control milk-borne staphylococcal intoxication, effcient storage conditions of milk and dairy products are the key step for to minimize the risk of staphylococcal food poisoning. For controlling *S. aureus* milk and milking environment adopting assurance quality systems are required in dairy industry (Cusato et al. 2014).

Although classical enterotoxins are the mainly isolated from staphyloccocal food poisoning, SEls can also cause outbreaks and intoxications. Umeda et al. (2017) were reported an outbreak from Japan caused by new SE/SEls and these findings indicated

SEA, *SEB*, *SEC*, *SED* and *SEE* types of staphylococcal enterotoxins are defined as the classical enterotoxins. Classical enteroxins have emetic activity which are associated with most of food poisoning caused by staphylococcal enterotoxins (Riva et al. 2015, Keyvan and Ozdemir 2016) These toxins have emetic activity and are usually associated with outbreaks of food poisoning (Le Loir et al. 2003). In this study, classical enterotoxins were detected by Ridascreen and *S. aureus* isolates from bulk tank milk contained classical enterotoxins genes. *seb* and *sec* gene were found as 16.6% (3) of *S. aureus* isolates (18).

that new SE/SEls can be the potential reason of staphylococcal intoxitacions.

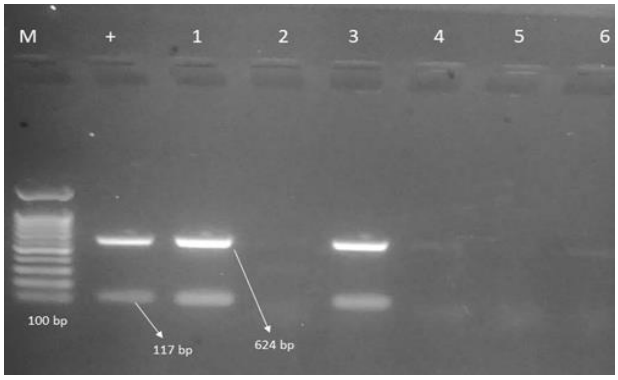

Figure 2. *coa*, *nuc* gene positive *S. aureus* isolates. M: Marker, +: *nuc* and *coa* gene positive *S. aureus* (ATCC 25923)

Şekil 2. *coa*, *nuc* geni pozitif *S. aureus* izolatları. M: Marker, +: *nuc* ve *coa* geni pozitif *S. aureus* (ATCC 25923)

In conclusion, milk is generally get contaminated by several microorganisms. Effective milk hygiene practices and good milking environment conditions should be provided by supplier in milk industry.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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