

***Staphylococcus aureus* and Staphylococcal enterotoxin detection in raw milk and cheese origin coagulase positive isolates**

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Abstract

In the present study, a total 110 (60 cheese and 50 cows' raw milk) samples was analyzed for coagulase-positive staphylococci (CPS) according to Food Drug Administration (FDA, 2001) previously. After the isolation, to confirmation of the isolates, catalase test, microscopic examination, coagulase test in tube and glucose-mannitol fermentation tests were applied. After the tests, we obtained 97 CPS isolates, and they were used as a material. We aimed that, 16S rRNA, nuc gene, and SEs genes in the *S.aureus* isolates were determined by using Polymerase Chain Reaction (PCR) assay. For the confirmation of the isolates being Staphylococci species, 16S rRNA was detected by using PCR assay. For the detection of the CPS isolates being *S. aureus*, nuc gene detected in the CPS isolates using PCR assay. The 16 S rRNA was detected in a total 97 (35 milk origin and 62 cheese origin) isolates. Therefore, these isolates were evaluated as (CPS). The nuc gene was detected in 50 out of 97 CPS isolates. So, the 50 (18 cheese origin and 32 milk origin) isolates were evaluated as *S. aureus*. However, none of the staphylococcal enterotoxin genes (SE A,B,C,D,E,G,H,I,J) was detected in 97 CPS or 50 *S. aureus* isolates.

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1. Introduction

Staphylococcal food poisoning is one of the leading foodborne illnesses in humans worldwide and is associated with contaminated foods of animal origin, such as milk and dairy products and other protein rich animal origin foods such as ice heavy cream, meat, poultry and fish (Tasci et al., 2011; Janstova et al., 2012). Several studies have shown that 15% to

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80% of the *S. aureus* isolated from various sources is able to produce enterotoxin (SE) (Toubar et al., 2018).

SEs are single polypeptides of approximately 26.900-29.600 kDa. To date, 23 SEs have been reported in literature (Ono *et al.*, 2015). SEA is the enterotoxin most frequently associated (Argudin et al., 2010) with staphylococcal foodborne outbreaks followed by SED. In Korea, about 90% of food poisoning isolates were reported to contain the *sea* gene (Cha *et al.*, 2006). SEA also was the most common SE associated to SFP in Japan (Shimizu *et al.*, 2000). In this country, an extensive outbreak that occurred in 2000 was attributed to low-fat milk containing SEA (Asao *et al.*, 2003). The SEA is produced throughout the log phase, while SEB, SEC, and SED are produced primarily during the transition from the exponential to the stationary phases of growth. Expression of SEB, SEC, and SED is affected by accessory gene regulator (*agr*), while SEA expressed simultaneously with the σ^{70} -like factor (Toubar et al., 2018).

The amount of enterotoxin necessary to cause intoxication is very small about 94-184 ng. The importance of the enterotoxins comes due to their heat stability and their resistance to inactivation by gastrointestinal proteases like pepsin (Rall et al., 2008). SEs are resistant to inactivation by gastrointestinal proteolytic enzymes, such as trypsin and pepsin. The enterotoxins are quite heat resistant and the heat stability is very important property of SEs in terms of food poisoning (Le Loir et al., 2003; Presscott et al., 2012; Toubar et al., 2018). Although *Staphylococcus* can be killed at normal cooking temperature, the toxins remain active (Le Loir et al., 2003). They retain their biological activity even after pasteurization; staphylococcal enterotoxin A (SEA), for example, keeps some activity after 28 min at 121°C (Rall et al. 2008).

The presence of a *S. aureus* enterotoxigenic strain places in the nasopharyngeal or oropharyngeal tract of a food handler (Todd *et al.*, 2010; Gallina *et al.*, 2013). Food poisoning occurs after the ingestion of food contaminated with enterotoxins produced by *S. aureus*; the onset of symptoms occurs a few hours (2–8) after ingestion of the contaminated food and improper preparation, handling, or storage (Schelin et al., 2011). Nausea, vomiting, abdominal cramp, and diarrhea are the most relevant symptoms (Riva et al. 2015); the disease severity depends on the amount of the ingested toxin and health of the consumer. In most

cases after 24 h, there is remission of symptoms; only a few cases of intoxications, ranging from 0.03% to 4.4%, are fatal in children and in the elderly (Doyle et al., 2007).

S. aureus is one of the ubiquitous microorganisms in the environment and can be found in the air, water, humans and animals. In 2015, 16 Member States (MS) reported 434 foodborne outbreaks caused by staphylococcal toxins (EFSA and ECDC, 2016). It is also one of the major causes of bovine mastitis and therefore, raw milk and subsequently raw milk products may be contaminated with *S. aureus* (Waage et al., 1999). About 10% of cheeses in Europe are made from raw milk, which presents a considerable potential threat to human health (Beuvier et al., 2004). For example, in Scotland, *S. aureus* was found to be the most frequent pathogen of raw milk cheeses in Scotland (Williams et al., 2010), and in France, a study of foodborne disease outbreaks showed that *S. aureus* was one of the most common causative pathogens associated with milk-related outbreaks (De Buyser et al., 2001). *S. aureus* may be introduced to bulk milk either by direct excretion from the udder of a cow with clinical or subclinical staphylococcal mastitis or by fecal contamination (Callon et al., 2008). *S. aureus* may be introduced to bulk milk either by direct excretion from the udder of a cow with clinical or subclinical staphylococcal mastitis or by fecal contamination (Callon et al., 2008). The other possible explanations of the higher *S. aureus* level in some raw milk samples may be their contamination from the milking equipment or personnel involved in production (Rola et al., 2016). Therefore, the aims of the study were to detect *S. aureus* and staphylococcal enterotoxin types in coagulase positive staphylococci isolates originated from cows' raw milk and cheese origins.

2. Materials and Methods

In the present study, a total 110 (60 cheese and 50 cows' raw milk) samples, consumed in Amasya province, Turkey, were analyzed for CPS according to Food Drug Administration (FDA, 2001) previously. After the isolation, to confirmation of the isolates, catalase test, microscopic examination, coagulase test in tube and glucose-mannitol fermentation tests were applied. After the tests, we obtained 97 CPS isolates. The 97 CPS isolates were used as a material. For the confirmation of the isolates being *Staphylococci* species, 16S rRNA was detected in the isolate according to by McClure et al. (2006) using PCR assay. For the detection of the CPS isolates being *S. aureus*, *nuc* gene detected in the CPS isolates according to McClure et al. (2006) using PCR assay.

16S rRNA PCR mixture and amplification programe: 25 µl final volume containing 1X PCR buffer (750 mMTris-HCl, 200 mM (NH₄)₂ SO₄), 3 mM MgCl₂, 200 µM dNTP, 0.07 µM 16S rRNA primer, 1U Taq DNA polymerase and 5 µM template DNA. The thermocycling conditions set at 94 °C for 10 min followed by 10 cycles of 94°C for 45 s, 55 °C for 45 s and 72°C for 75 s, and 72°C for 10 min. After the amplication, 100 bp DNA ladder mix (Thermo Scientific) was used to provide molecular size markers. They were loading 2% agarose gels with ethidium bromide (5 µg /ml) and electrophoresis procedure was applied.

nuc gene PCR mixture and amplification program: For this aim, PCR procedure was applied according to Louie et al. (2002); 25 µl final volume containing 1X PCR (750 mMTris-HCl, 200 mM (NH₄)₂ SO₄), 3 mM MgCl₂, 200 µM dNTP, 0.2 µM nuc primer, 1U Taq DNA polymerase and 5 µM template DNA. The thermocycling conditions set at 94 °C for 2 min followed by 25 cycles of 94°C for 15 s, 55 °C for 30 s and 72°C for 30 s, and 72°C for 10 min. After the amplication, products were loading 2% agarose gels with ethidium bromide (5 µg /ml) and electrophoresis procedure was applied. The primer sequence have shown in Table 1.

Table 1. Primer sequence used in the present study

Target Gene	Oligonucleotide sequence	Product size (bp)	References
16SrRNA <i>Staphylococcus</i>	F AAC TCT GTT ATT AGG GAA GAA CA R CCA CCT TCC TCC GGT TTG TCA CC	756	Zhang et al., 2004
<i>S.aureus/ nuc</i>	F GCG ATT GAT GGT GAT ACG GTT R AGC CAA GCC TTG ACG AAC TAA AGC	279	Louie <i>et al.</i> , 2002
SEA/ <i>sea</i>	F GCA GGG AAC AGC TTT AGG C R GTT CTG TAG AAG TAT GAA ACA CG	521	Monday&Bohach, 1999
SEB/ <i>seb-sec</i>	F ACA TGT AAT TTT GAT ATT CGC ACTG R TGC AGG CAT CAT GTC ATA CCA	667	Lovseth et al., 2004
SEC/ <i>sec</i>	F CTT GTA TGT ATG GAG GAA TAACAA R TGC AGG CAT CAT ATC ATA CCA	284	Monday&Bohach, 1999
SED/ <i>sed</i>	F GTGGTG AAA TAG ATA GGA CTG C R ATA TGA AGG TGC TCT GTG G	385	Monday&Bohach, 1999
SEE/ <i>see</i>	F TAC CAA TTA ACT TGT GGA TAG AC R ATA TGA AGG TGC TCT GTG G	171	Monday&Bohach, 1999
SEG/ <i>seg</i>	F CGT CTC CAC CTG TTG AAG G R CCA AGT GAT TGT CTA TTG TCG	328	Monday&Bohach, 1999
SEH/ <i>seh</i>	F CAA CTG CTG ATT TAG CTC AG R GTC GAA TGA GTA ATC TCT AGG	359	Monday&Bohach, 1999
SEI/ <i>sei</i>	F CAA CTC GAA TTT TCA ACA GGT AC R CAG GCA GTC CAT CTC CTG	466	Monday&Bohach, 1999
SEJ/ <i>sej</i>	F CAT CAG AAC TGT TGT TCC GCT AG R CTG AAT TTT ACC ATC AAA GGT AC	142	Monday&Bohach, 1999

*SE, Staphylococcal Enterotoxin

SEs detection in the isolates: SEs detection in the *S. aureus* and other CPS isolates were applied according to Monday&Bohach (1999) using PCR assay. The PCR mixture and amplicon program have shown in Table 2 and Table 3, respectively.

Table 2. Primer sets: SEs determination in the *S. aureus* isolates

Master mix Compounds	Volume in Microliter
PCR buffer 10X	2 µl
MgCl ₂ (25 mM)	2 µl
dNTP (10 mM)	1.2 µl
Forward Primer	0.75 µl
Reverse Primer	0.75 µl
Taq Pol. Enzyme (5 U/ µl)	0.4 µl
DNA sample	2 µl
DDW (double distile water)	10.9 µl
Total volume for each reaction	20 µl

Table 3. PCR condition program for SEs determination in the *S. aureus* and other CPS isolates

PCR Sections	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
Number of Cycle	1	30			1
Temp. in celsius	95	95	63	72	72
Duration in minute	10	1	1	1	10

3. Results and Discussion

In the present study, 60 cheese and 50 raw milk samples were analyzed for present of *S. aureus*. A total 97 isolates were obtained. According to 16 S rRNA determination result by using PCR assay (Figure 1), the gene was detected in a total 97 (35 milk origin and 62 cheese origin) isolates. Therefore, these isolates were evaluated as coagulase-positive staphylococci (CPS). For determination of *S. aureus*, *nuc* gene was determined by using PCR assay (Figure 2). The gene was detected in 50 out of 97 CPS isolates. So, the 50 (18 cheese origin and 32 milk origin) isolates containing *nuc* gene were evaluated as *S. aureus*. To determination of staphylococcal enterotoxin determination, PCR assay was applied. According to PCR assay result, none of the staphylococcal enterotoxin genes (SE A,B,C,D,E,G,H,I,J) was detected in 97 CPS or 50 *S. aureus* isolates.

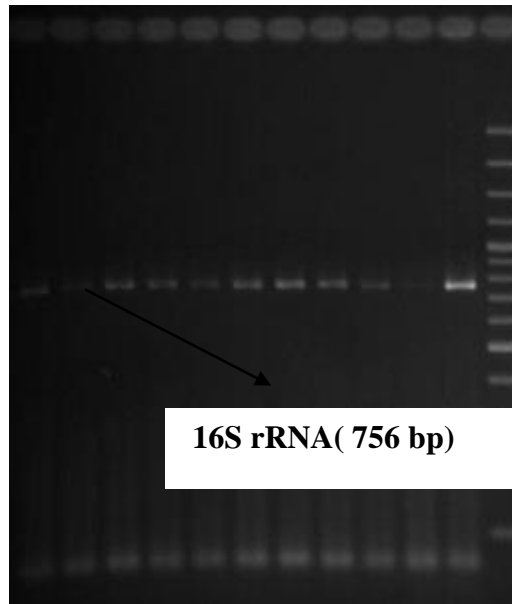


Figure 1. 16S rRNA detection in CPS isolates

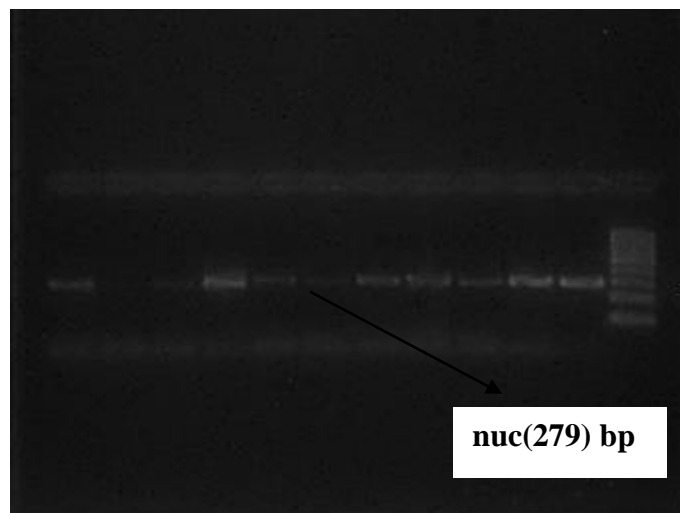


Figure 2. *nuc* gene detection in the CPS isolates

The present study, although *S. aureus* was detected in 50 (18 cheese and 32 milk origin) out of 97 CPS isolates, none of the isolates don't have any of the SEs genes used in the study. In addition, although milk origin isolates number higher than cheese origin, the contamination level of CPS in the cheese samples higher than the milk samples.

There have been some studies according to staphylococcal food poisoning after consumption of unpasteurized milk or cheese samples in different countries around the world (Simeão do Carmo et al., 2002; Ostryn et al., 2009; Carfora et al. 2015; Rola et al., 2016). For the cheese-borne staphylococcal food poisonings, from Brazil, Simeão do Carmo et al. (2002) reported

that after Minas cheese consumption, staphylococcal food poisoning was seen in 378 people, and of 50 people of them became ill. In the other staphylococcal food poisoning, responsible food was raw milk. After the consumption of the milk, 328 people were affected. In the study results, researchers also reported that the two food poisoning, raw milk and cheese samples were contaminated with *S. aureus* at levels of 10^3 - 10^8 cfu/g, and SEA, SEB and SEC type toxins detected in the *S. aureus* isolates. In the study results, workers hand and mastitis were responsible source of *S. aureus*. In the report, after the cheese consumption, it was seen staphylococcal food poisoning, of 26, 23 individuals were affected the illness due to the SEE producing *S. aureus*. From Poland, Rola et al. (2016) reported that 244 cheeses made from raw milk samples were analyzed for *S. aureus*, enterotoxin genicity and resistance to antimicrobials of *S. aureus*. They found that 122 (50.0%) of the samples were contaminated with *S. aureus*, based on the *nuc* gene, including 18 of 26 (69.2%) mature cheese samples with log₁₀ CFU g⁻¹ between <1- and 7.41. ELISA test was applied for the presence of SEs and all of them were negative. However, SE genes were found in 55 (45.1%) isolates. Most of them were positive for three enterotoxin markers, i.e., *sed* + *sej* + *ser* and *sec* + *seg* + *sei* (26 and three isolates, respectively). Another study, Carfora et al. (2015) reported from Italy that a total of 227 *S. aureus* colonies, isolated from 54 samples of raw milk and dairy products of bovine, ovine, caprine and bubaline origin were tested for the presence of genes coding for SE (SEs/SEIs) and for methicillin resistance. Ninety-three colonies, from 31 of the 54 samples (57.4%) and from 18 (69.2%) of the 26 farms of origin tested positive for SEs/SEIs genes. Most of the isolates harbored more than one toxin gene and 15 different toxin types were recorded. The most frequent were “*sec*” gene alone (28.6%), “*sea, sed, ser, selj*” (20%), “*seg, sei*” and “*seh*” (8.6%). The 77 colonies harboring “classical enterotoxins” genes (*sea-sed*) were further tested for enterotoxin production, which was assessed for 59.2% of the colonies.

There have been many studies about contamination of milk and cheese samples with *S. aureus* and their staphylococcal enterotoxin. From Iran, for example, it was reported that *S. aureus* was isolated from 200 organic milk (n=100) and cheese (n=100) samples at 27% ratio, and SEA gene was detected in 12.96 % of *S. aureus* isolates (Saadat et al. 2014). Another study reported from Italy that Carfora et al. (2015). In the study, *S. aureus* was isolated from 54 samples and total 227 *S. aureus* colonies were obtained from different source of milk and dairy products, and 93 isolates were capable of 15 different staphylococcal enterotoxins. The most frequent were “*sec*” gene alone (28.6%), “*sea, sed, ser, selj*” (20%), “*seg, sei*” and “*seh*” (8.6%). They concluded that the obtained results indicated that the analyzed cheeses were safe

for consumers. To improve the microbiological quality of traditional cheese products more attention should be paid to animal welfare and hygienic practices during the process of cheese manufacturing in some dairy farms.

These results mentioned above confirm that enterotoxigenic *S. aureus* can be commonly found in milk and dairy products, as reported in other studies conducted in Northern Italy (Bianchi et al., 2014) and Switzerland (Hummerjohann et al., 2014).

From Turkey, there have been several studies from different regions. One of them, Yücel and Anıl (2011) reported that CPS was isolated from 236 raw milk and cheese samples. *S. aureus* was also detected in 35% ratio of CPS. Another study was reported from Istanbul. In the study, Gökmen et al. (2013) reported that *S. aureus* was detected in 50 out of 150 different cheese samples. Enterotoxin was also determined in 25 samples.

The present study results different from above studies results reported from different countries. Hummerjohann et al. (2014) and Normanno et al. (2007) reported that many of their analyzed samples yielded both SEs/SEIs-positive and SEs/SEIs-negative colonies at the same time. For the reason, they underlined the importance testing more than one colony per sample. Using the PCR approach, the possibility of testing more colonies or even colony pools to increase the chances of detecting positive samples could be taken into account. The present study, enterotoxigenic *S. aureus* isolates might be present in the analyzed samples if would analyzed more isolates. Another reason may be used different primer sequence. So, there can be differences in term of spasticity and sensitivity of different primers.

It is known that heat treatment has not sufficient for elimination or destruction of SEs. According to the matter, Necidova et al. (2016) examined the effect of pasteurization inactivating staphylococcal enterotoxins A, B, and C in milk. For this, milk samples were inoculated with log 4.38 to 5.18 cfu/ml of 50 different *S. aureus* strains having the ability to produce types A, B, or C SE and incubated at 37°C for 24 h to develop SE. This incubation was followed by heat treatment for 15 s at 72, 85, and 92°C. Samples were analyzed for *S. aureus* count by plate method and, specifically, for SE presence. As a result, they found that the *S. aureus* count in milk before pasteurization did not affect the amount of SE. Before pasteurization, SEB was detected in the lowest amount compared with other SE types. Staphylococcal enterotoxins were markedly reduced with pasteurization and inactivated at

pasteurization temperatures to an extent depending on the amount in the sample before pasteurization. After pasteurization at 72°C, SE were detected in 87.5% of the samples, after pasteurization at 85°C in 52.5% of the samples, and after pasteurization at 92°C in 45.0% of the samples. We determined that SE may still persist in milk even when *S. aureus* bacteria are inactivated through pasteurization. Although pasteurization may partially inactivate SE in milk, a key measure in the prevention of staphylococcal enterotoxigenesis linked to pasteurized milk consumption is to avoid any cold chain disruption during milk production and processing. In another study, Medved'ová et al. (2009) study results showed that growth of *S. aureus* and SED production in milk were 12 °C to 21 °C at temperatures. SED was detected at the level of *S. aureus* 10⁶ cfu /ml at the lower temperature of 12 °C. At the higher temperatures of 18 °C and 21 °C, SED was detected when *S. aureus* reached the counts of 10⁷ cfu /ml. The present study, maximum CPS levels was 10⁴ cfu/ml in 4% of the raw milk samples, and 60% of the samples were contaminated with CPS at level of 10²-10³ cfu/ml. Therefore, the CPS levels were quite lower for SE production. Most likely, *S. aureus* contamination levels were lower than the CPS levels. However, the present study, the maximum contamination level of 13% of cheese samples was 10⁶-10⁷ cfu/g. The levels were higher than the cheese samples. Therefore, compared the milk samples, the cheese samples have more risk for humans in terms of CPS, *S. aureus* and their SE toxins. According to Charlier et al. (2008) study results have shown that the growth of *S. aureus* in raw milk was clearly lower in comparison with pasteurized milk. The reason is the natural microflora of raw milk that can inhibit *S. aureus* growth and thus also SE production. Janštová et al. (2014) also reported that pasteurized milk was a good substrate for enterotoxin production. At 15 °C, SEC was detected within 48 h of incubation in ewes' and goats 'milk, and within 24 h of incubation in cows' milk. At 22 °C, enterotoxin production was detected as early as within 24 h of incubation. According to the results, incubation temperature of the cheese samples also important for growth of *S. aureus* and SE production.

In conclusion, according to analyzed result reported from around the world, reported results emphasize the need for better hygienic practices throughout the production, processing, and marketing of dairy products. If raw milk is used for cheese making, the acidification process is of the most importance. For determination SE genes in the CPS or *S. aureus* isolates, at least two different primer sets should be used.

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