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Survey on Methicillin Resistance and Panton Valentine Toxin of *Staphylococcus aureus* Strains Isolated from Raw Milk and Ice Cream: Molecular Study by Multiplex PCR

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Abstract

Staphylococcus aureus is a very important pathogenic bacterium that causes nosocomial and community-acquired infections in humans, and is also one of the leading pathogens that causes food-borne poisoning. The presence of *S. aureus* in raw milk and dairy products, and especially the presence of MRSA (Methicillin Resistance *S. aureus*) strains, poses a potential risk to public health. The aim of this study was to investigate the presence of methicillin resistance and Panton-Valentine Leucocidin (PVL) toxin in *Staphylococcus aureus* isolated and identified from raw milk and ice cream in Konya (Turkey) by multiplex PCR method. A total of 55 *S. aureus* were isolated 49 (18%) from 260 raw milk samples collected from various farms and 6 (4%) from 150 ice cream samples sold in patisseries. The obtained isolates were identified as *S. aureus* with conventional and genotypic methods. Multiplex PCR was performed to detect the 16S rRNA, *mec*A, *fem*A and *luk*S genes. While no *mec*A gene was detected in any of the 49 *S. aureus* isolates obtained from raw milk samples, the presence of *mec*A gene was observed in one of the 6 *S. aureus* isolates isolated from ice cream samples. The PVL gene was not detected in any of the *S. aureus* isolates studied. *S. aureus* contamination is common in raw milk samples and ice cream samples. In order to avoid this, it is necessary to comply with the hygiene conditions and increase the precautions even more.

Çiğ Süt ve Dondurmadan İzole Edilen *Staphylococcus aureus* Suşlarının Metisilin Direnci ve Panton Valentine Toksini Üzerine Araştırma: Multiplex PCR ile Moleküler Çalışma

Anahtar kelimeler
MRSA; Panton-Valentine Lökosidin; lukS; mecA; Süt Ürünleri

Öz

Staphylococcus aureus insanlarda hastane ve toplum kökenli enfeksiyonlara neden olan çok önemli bir patojen bakteridir ve aynı zamanda gıda kaynaklı zehirlenmelere neden olan patojenlerin başında gelmektedir. Çiğ süt ve süt ürünlerinde *S. aureus* ve özellikle MRSA (Metisilin Direnli *S. aureus*) suşlarının varlığı halk sağlığı için potansiyel bir risk oluşturmaktadır. Bu çalışmanın amacı, Multiplex PCR yöntemiyle, Konya'da (Türkiye) çiğ süt ve dondurmadan izole ve identifiye edilen *Staphylococcus aureus*'larda metisilin direnci ve Panton-Valentine Lökosidin (PVL) toksini varlığının araştırılmasıdır. Çeşitli çiftliklerden toplanan 260 çiğ süt örneğinden 49 (%18) ve pastanelerden toplanan 150 dondurma örneğinden 6 (%4) adet olmak üzere toplam 55 *S. aureus* izole edildi. Elde edilen izolatlar, konvansiyonel ve genotipik yöntemlerle *S. aureus* olarak tanımlandı. 16S rRNA, *mec*A, *fem*A ve *luk*S genlerini saptamak için multipleks PCR yapıldı. Çiğ süt örneklerinden elde edilen 49 *S. aureus* izolatının hiçbirinde *mec*A geni saptanmazken, dondurma örneklerinden izole edilen 6 *S. aureus* izolatından birinde *mec*A geni varlığı belirlendi. PVL geni, çalışılan *S. aureus* izolatlarının hiçbirinde saptanmadı. *S. aureus* kontaminasyonu çiğ süt numunelerinde ve dondurma numunelerinde oldukça yaygındır. Bu kontaminasyonun önüne geçebilmek için hijyen koşullarına uyulması ve önlemlerin daha da artırılması gerekmektedir.

1. Introduction

Staphylococcus aureus, belonging to the Micrococcaceae family, is a Gram-positive, facultative anaerobic, non-spore-forming, non-motile, catalase-positive bacterium. It is naturally found in the upper respiratory tract and skin of humans and animals microbiota (Issa and Aksu 2020, Kramer *et al*. 1989). Some strains of *Staphylococcus aureus* are highly pathogenic and can cause various diseases in humans and animals. A significant public health problem related to staphylococcal infections in recent years is the widespread use of antimicrobials in human and veterinary medicine, as well as in animal husbandry and other agricultural activities. As a result of the excessive use of antimicrobial agents, the number of *S. aureus* strains with drug resistance is increasing day by day. (Riva *et al.* 2015, Titouche *et al.* 2019). Methicillin-resistant *S. aureus* (MRSA) strains are very important due to their high morbidity and mortality. The presence of the *mec*A gene, which encodes a modified penicillin-binding protein (PBP2a) with low affinity for β-lactams is responsible for the methicillin and antibiotic resistance to other beta-lactam groups antibiotics. The *mec*A gene is localized on a mobile genetic element called the staphylococcal cassette chromosome mec (SCCmec) (Titouche *et al*. 2019, Visciano *et al.* 2014).

S. aureus is one of the most common food-borne pathogens and is the leading reason of food-related epidemics worldwide (Paterson *et al*. 2012). MRSA causes mastitis in livestock and can contaminate milk and dairy products. Mastitis adversely affects the health of the animal and also reduces the quantity and quality of milk production. However in recent years, it has become quite worrying that methicillin resistance can be transferred to humans through the consumption of these products (Asiimwe *et al*. 2017, Fagundes *et al*. 2010, Keyvan *et al*. 2020). Raw milk is an ideal reservoir for the development of microorganisms. Milk and dairy products produced under non-hygienic conditions play an important role in food poisoning and epidemics (Fagundes *et al*. 2010, Zecconi and Hahn 1999). *S. aureus* threatens human and animal health by the enzymes and toxins it produces, such as toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxin (SE) and Panton-Valentine leukocidin (PVL) (Wang *et al*. 2018). PVL is an important virulence factor, causing lysis by forming pores with its cytolytic effect on basophil leukocytes, monocytes and macrophages. Consequently PVL has a vital role of escaping *S.aureus* from the immune system. The clinical course of infections due to PVL positive *S.aureus* strains is more vigorous than PVL negative strains (Duman and Otlu 2013, Löffler *et al*. 2010). The main goal of the study was to define the presence of methicillin resistance and PVL toxin in *S. aureus* strains isolated and identified from raw milk and ice cream in Konya by multiplex PCR method.

2. Materials and Methods

2.1 Samples

In April 2014 -September 2015 periods, 260 milk samples from the milk producers in the center of Konya and its surroundings, and 150 ice cream samples sold in the local markets and patisseries in the city center were collected. Samples were stored in sterile plastic collection containers, transported to the laboratory with ice coolers (4-8 °C) and analyzed the same day.

2.2 Isolation and identification of S. aureus

Raw milk and ice cream samples (0.1 ml) were plated on Baird Parker agar (Merck) supplemented with egg yolk and tellurite emulsion (Merck) and Mannitol salt agar phenol red (Merck). Colonies forming a yellow halo in Mannitol salt agar phenol red medium were suspected to be *S. aureus*. Also, colonies on Baird Parker agar surrounded by grey to black (potassium tellurite reaction) and clear areas (egg yolk reaction) were considered to be *S. aureus*. Colonies suspected of being *S. aureus* were subjected to the following tests: coagulase test, catalase reaction, Gram staining and ß hemolysis. *S. aureus* ATCC 25923 (MSSA), *S. aureus* ATCC 29213 (MSSA), *S. aureus* ATCC 43300 (MRSA) and *S. epidermidis* NRLL B-4268 strains used as control strains were obtained from the Microbiology Research Laboratory of the Biology Department of the Faculty of Science, Selcuk University.

2.3. Multiplex PCR Assay

DNA extraction from *S. aureus* isolates was conducted using a commercial DNA extraction kit (Thermo Scientific Genejet Genomic DNA Purification Kit) as recommended by the manufacturer. Genotypic identification of the isolates was determined using specific primers for the Staphylococcus genus-specific 16S rRNA gene region and the *S. aureus* specific *fem*A gene region (Table 1). In addition, the *mec*A gene region was used to identify the methicillin resistance and the lukS gene region was used to determine the pvl gene of *S. aureus* isolates. The method of multiplex PCR described by Al- Talib *et al*. (2009) was used with several modifications. These region-specific primers are given in Table 1. PCR amplification for a sample was prepared in a total volume of 50 µl. For each sample, 5 µl DNA polymerase buffer (10x), 4 µl MgCl2 (25 mM), 1.5 µl dNTP (10mM), 4 µl BSA (100x), 1.5 µl Taq polymerase (5µl), 1 µl reverse and forward primers (20mM) and 3 µl of DNA (200ng / µl) were added and finally the desired volume was prepared with ultrapure water. The PCR was carried out using a Mastercycler Gradient (Thermo) with one cycle of initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 60°C, and extension at 72°C for 30 s, followed by an extra cycle of annealing at 60°C for 30 s, and a final extension at 72°C for 5 min. The PCR products were analyzed by electrophoresis on 1.5% low EEO agarose gels, with ethidium bromide at 100 V for 75 min. PCR products were visualized under UV illumination and photographed using an image analyzer (BioRad).

Table 1. Primer sequences, target gene region and amplicon sizes used in multiplex PCR (Al-Talib *et al.* 2009).

Primers	Primer sequences 5'- 3'	Target Gene	Amplicon size (bp)
16S rRNA-F	GCAAGCGTTATCCGGATTT	16S rRNA	597 bp
16S rRNA-R	CTTAATGATGGCAACTAAGC		
femA-F	CGATCCATATTTACCATATCA	*femA*	450 bp
femA-R	ATCACGCTCTTCGTTTAGTT		
mecA-F	ACGAGTAGATGCTCAATATAA	*mecA*	293 bp
mecA-R	CTTAGTTCTTTAGCGATTGC		
lukS-F	CAGGAGGTAATGGTTCATTT	*lukS*	151 bp
lukS-R	ATGTCCAGACATTTTACCTAA		

3. Results

In our study, 260 raw milk samples and 150 ice cream samples were examined in terms of *S. aureus* carrier. According to the data we obtained as a result of phenotypic and genotypic tests; a total of 55 *S. aureus* were isolated from 49 (18%) raw milk samples and 6 (4%) ice cream samples (Table 2). Multiplex PCR was successfully optimized to simultaneously identify 16S rRNA for *Staphylococcus*, femA specific for *S. aureus*, *mec*A for methicillin resistance, and *luk*S for PVL toxin. In the multiplex PCR method, 16S rRNA and *fem*A gene regions were determined in all 55 *S. aureus* isolates, which were defined by phenotypic methods (Figure 1 and 2). Also, *mec*A gene was not determined in any of the 49 *S. aureus* isolates obtained from raw milk samples, ın contrast the *mec*A gene was detected in one of the 6 *S. aureus* isolates (GS 55: İce cream isolate) isolated from ice cream samples. Therefore, only one (1.8%) of 55 foodborne *S. aureus* isolates was detected as MRSA. Furthermore, It was determined that none of the 55 *S. aureus* strains isolated from raw milk and ice cream samples harbored the gene encoding PVL (Figure 1 and 2).

Table 2. Total number of food samples and *S. aureus* isolates.

Sample type	Total number of samples	Total number of *S. aureus* isolates
Raw milk	260	49 (%18)
Ice cream	150	6 (%4)

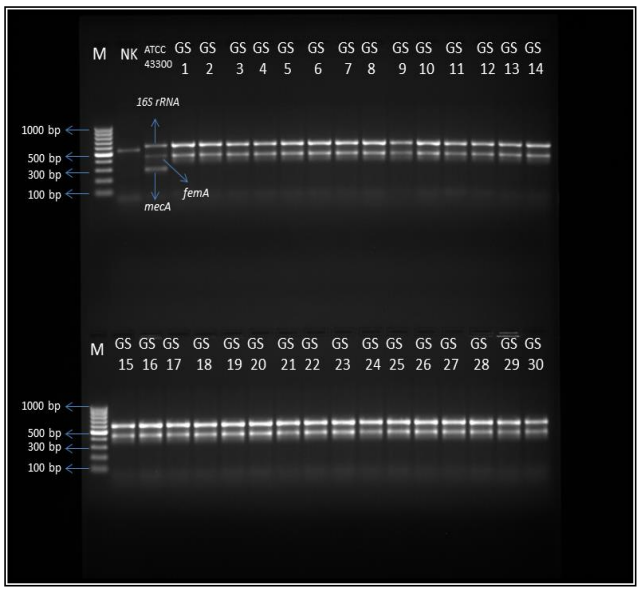

Figure 1. Multiplex PCR assay profile with *S. aureus* isolates and reference strains (Respectively M: Marker, NK: Negative control *S. epidermidis* NRRL B-4268, S. aureus ATCC 43300 (MRSA), GS 1-30: *S. aureus* isolates isolated from raw milk)

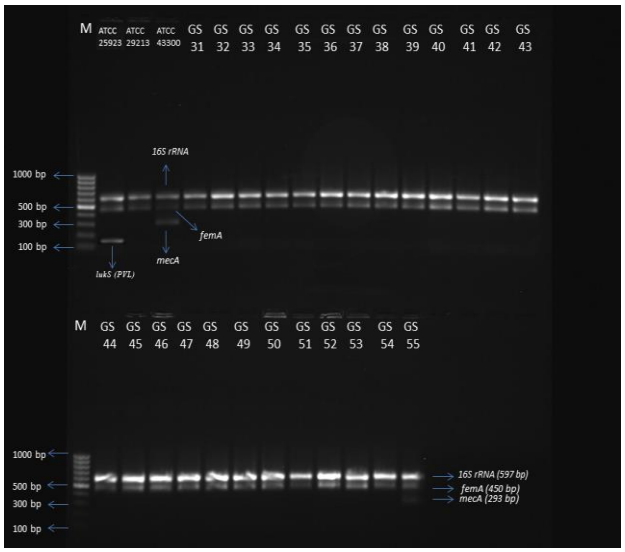

Figure 2. Multiplex PCR assay profile with *S. aureus* isolates and reference strains (Respectively M: Marker, *S. aureus* ATCC25923 (MSSA), *S. aureus* ATCC29213 (MSSA), *S. aureus* ATCC 43300, GS 31-49: *S. aureus* isolates isolated from raw milks, GS 50-55: *S. aureus* isolates isolated from ice creams)

4. Conclusion and Discussion

S. aureus is a very important pathogenic bacterium that causes nosocomial and community-acquired infections in humans, and is also one of the leading pathogens that cause foodborne poisoning. MRSA isolates that emerged shortly after the introduction of methicillin have become a growingly important health problem in Turkey and all over the world in recent years, as they increase mortality and morbidity in patients with MRSA infection (Ichiyama *et al*. 1991, Riva et al. 2015). The presence of *S. aureus* and especially MRSA strains in raw milk and dairy products and the possibility of spreading these microorganisms by consuming these foods are important for public health (McKay 2008). Although the role of food in MRSA infections in humans today is of secondary importance, it should not be ignored that these strains can evolve rapidly and their virulence and contagiousness can change (Riva *et al*. 2015). Due to these changing characteristics, MRSA strains may have the potential to be easily transmitted from animals to humans. Our study aimed to determine the presence of methicillin resistance gene and PVL toxin in *S. aureus* strains isolated from raw milk and ice cream samples by multiplex PCR method.

There are different studies conducted on this subject in our country. Issa and Aksu (2020) determined the rate of *S. aureus* as 29.31% in their research on raw milk samples. They identified only one of the isolated *S. aureus* strain as MRSA. The rate of *S. aureus* in raw milk detected in our study (18%) is lower than the rate found by the researchers, and methicillin resistance was not detected in any of these strains. Aydın *et al*. (2011) detected *S. aureus* strain in 31 (10.2%) of 303 raw samples collected in the Marmara region. In our study, *S. aureus* strain was detected in 49 (18%) of 260 raw milk samples. The rate we found is higher than the rate found by the researchers. When we examined different studies on the prevalence of *S. aureus* in raw milk in our country, the rates of *S. aureus* strains detected in raw milk were reported as 14%, 33.3%, 35% and 64% (Ertaş and Gönülalan 2010, Guven *et al*. 2010, Yildirim *et al*. 2019, Yücel and Anıl 2011). In another study conducted with multiplex PCR, the prevalence of *S. aureus* was defined as 75 % (Gücükoğlu *et al*. 2012). Comparing with our rates, the results the researchers found were quite high. The different rates reported in studies suggest that milking conditions and the hygienic quality of milk may differ. In the presented study, methicillin resistance was not detected in any of the *S. aureus* strains isolated from raw milk.

Similarly, Can *et al*. (2017) and Ektik *et al*. (2017) did not detect methicillin resistance in *S. aureus* strains isolated from raw milk in their studies.

Similar studies were also conducted in other countries. In a study performed in Brazil, 18 (7.3%) *S. aureus* strains were isolated from 245 raw milk samples (Fagundes *et al*. 2010). In another study presented in Brazil, 20 (4%) *S. aureus* was isolated from 473 milk samples and no *mecA* gene was determined in any isolates *(*Martins *et al*. 2015). Similarly, 143 raw milk samples were collected in Mozambique and 58 (41%) *S. aureus* was isolated. They found that only 2 (3%) of the isolates were MRSA (Nhatsave *et al*. 2021). Again, the prevalence of *S. aureus* was reported as % 19.8 in India, 21% in Iran, 32.5 % in Poland 41% in China and 53.5% in Italy (Giacinti *et al*. 2017, Korpysa-Dzirba and Osek 2011, Nazari *et al.* 2014, Sharma *et al*. 2017, Wang *et al*. 2018).

In our presented study, 6 (4%) *S. aureus* was isolated from 150 samples of ice cream and only one strain was *mecA* positive. In some studies carried out with ice cream in our country, different rates of *S. aureus* strains were detected (Ağaoğlu and Alemdar 2004, Keskin *et al*. 2007). In a study conducted in Istanbul, 101 ice cream samples sold in markets were collected and *S. aureus* was isolated in 66 (65.3%) of these samples (Ede 2016). This reported rate is quite high compared to the rate we found in our study (4%). In the study of Gücükoğlu *et al*. (2012), the presence of *mecA* gene was reported in only one of 35 *S. aureus* isolated from ice creams. Similarly, the presence of *mecA* gene was determined in only one isolate in our study. In a study conducted in China on ice creams, *S. aureus* was detected in 13 (5.4%) of 240 ice cream samples, and also the *mecA* gene was determined in two of these isolates (Zhang *et al*. 2021). Our rates are consistent with the researchers' results. In another study performed in Egypt, *S. aures* was isolated in 15 of 100 ice cream samples and the *mecA* gene was determined in 10 of these isolates (Samir *et al*. 2018). Foods that are sold in the open area, especially ice cream, which is consumed a lot in the summer season, threaten human health. Detection of *S. aureus* in ice creams may have been due to faulty pasteurization of raw milk used in ice cream production, use of contaminated tools and equipment, and inadequate personnel hygiene. The fact that ice cream production in our country is mainly in the form of patisserie ice cream and selling of these ice creams in the open area causes an increase in the risk of contamination (Ağaoğlu and Alemdar 2004).

In our study, no PVL gene was detected in any of the food samples. Similarly, in the study of Riva *et al*. (2015) in Italy with raw milk samples, the PVL gene was not detected in any of the isolated *S. aureus*. In other studies on foods, similar to our results, no PVL gene was detected in any *S. aureus* isolate (Alizadeh and Amini 2015, Bonsaglia *et al*. 2018, Titouche *et al*. 2019). However, PVL rates of 4.1% and 14% have been reported in some studies (Basanisi *et al*. 2017, Nhatsave *et al*. 2021). The spread of PVL-positive strains, which have high morbidity and mortality, in food may pose significant risks to public health. Therefore, It is pleasing that no PVL positive strains were found in our study.

In conclusion, *S. aureus* strains were isolated from raw milk and ice cream samples, and the presence of *mecA* and PVL genes was determined in these isolates by the multiplex PCR method in our study. The presence of *S. aureus* in raw milk and dairy products, and especially the presence of MRSA strains, poses a potential risk for food poisoning. *S. aureus* contamination is common in raw milk samples and ice cream samples. In order to prevent this, we believe that it would be appropriate to comply with the hygiene conditions and to increase the precautions even more. Particular attention should be paid to both hygiene and the cold chain, especially in the stages from milking to the end consumer. Ice creams should be bought from reliable places and it is recommended to avoid the consumption of ice creams sold in the open area, if possible.

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