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Isolation and identification of *Staphylococcus aureus* obtained from cheese samples

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ABSTRACT

Milk and dairy products including cheese are one of the most significant food commodities in terms of the food industry. However, a contaminated food product could conduce a variety of food borne bacterial infections. Although *Staphylococcus aureus* is known as normal flora members of the humans, it`s often isolated from the community and hospital-acquired infections. Therefore, investigation of *Staphylococcus aureus* from cheese samples was aimed in this study. A total of nineteen (n=19) white cheese was collected from various outdoor markets in Istanbul. All cheese samples were evaluated quantitatively. Phenotypic identification tests including Gram staining, oxidase, catalase, mannitol, and DNase were performed. The presumptive *Staphylococcus aureus* colonies (n=47) were analyzed by the 16S rRNA PCR and sequencing. And the sequences were deposited into the National Center for Biotechnology Information. According to the nucleotide BLAST analysis, a total of 47 *Staphylococaceae and Enterococcaceae* members including *Staphylococcus aureus* (n=3), *Staphylococcus carnosus* (n=1), *Macrococcus caseolyticus* (n=1), *Enterococcus faecalis* (n=25), *Enterococcus faecium* (n=12*), Enterococcus durans* (n=4), *and Enterococcus gallinarum* (n=1) were identified. Regarding methicillin susceptibility testing, two of out of three *Staphylococcus aureus* were detected as methicillin-resistant.

Keywords: *Staphylococcus aureus*, 16S rRNA, cheese, PCR

Introduction

The white cheese is the most consumed cheese type in Turkey and the cheese consumption per capita was determined as 8.7 kg/person in 2017 and 9.2 kg in 2020 (Temelli et al.,2006; Ataseven, Z, 2017; www.statista.com) Cheese is such a nourishing food that could provide an environment to the bacteria for growing and multiplication including *Salmonella*, *Escherichia*, and *Staphylococcus* because of the contamination. From the production of cheese to the point of sale, an inadequate sanitization procedure of equipment and utensils lead to contamination of the cheese products and this affects not only food quality but also public health (Donnely, 1990; Aguilar et al., 2016).

Staphylococcus aureus (*S. aureus*) is known as normal flora member of the human skin, however, some strains of the *S. aureus* is the main reason of the infections and intoxications in terms of consumption of the contaminated milk, dairy products and other foods (Kadiroglu et al., 2014; Bingöl and Toğay, 2017). Staphylococcal food intoxication is a gastrointestinal disease that occurs due to the toxin produced *S. aureus*. When food or ingredients is contaminated by the enterotoxigenic strain of *Staphylococcus* spp., Staphylococcal food poisoning could be induced on the occasion of *Staphylococci* growth and enterotoxin production (Hennekinne et al., 2012; https://www.ndhealth.gov/Disease). Moreover, pathogenic strains of *S. aureus* could cause skin lesions, septicaemia, and meningitis in humans and it`s responsible for bovine mastitis in animals (Younis et al., 2003; Baran et al., 2017). The transmission of *S. aureus* to dairy products such as milk and cheese could occur via mastitis, mammary glands or animal, skin (Saka and Gulel, 2018). There may be a risk of contamination from personnel and equipment during the production of dairy products. In other words, transmission can be occurred also by animal to animal during milking as well as by the food-handlers, human to food contamination route (Kümmel et al 2016; Monte et al., 2018). Methicillin-resistant *S. aureus* (MRSA) is one of the most significant bacteria in terms of human global health due to the responsible for both community and hospital-acquired infections (Harrison et al., 2014). Moreover, livestock-associated MRSA (LA-MRSA) infections originated from livestock such as pigs, goats, and dairy cattle could transmit to the humans who is working in farms and abattoirs where raw meat processed. LA-MRSA could be occurred by handling contaminated meats. Therefore, LA-MRSA could be also the reason for human infections (Cuny et al., 2015).

Although, the isolation of the MRSA from animal and food origin were investigated frequently, the adverse effect of MRSA in dairy products illness is relatively low (Herrera et al., 2016). Hence, identification of *S. aureus* in cheese samples is important for both the food industry and public health. In this study, it was aimed to identify *S. aureus* in white cheese samples sold in outdoor markets in Istanbul.

Materials and Methods

Sample Collection and Bacteriological Analysis

A total of nineteen (n=19) white cheese was collected from outdoor markets in Istanbul in April 2018 and September 2019. The color and pH value of each cheese samples were recorded (Creamy and white, pH:6.8-7.5). The cheese samples were analyzed quantitatively by homogenizing 25 g cheese and 225 ml peptone water (Peptone:10 g/L, NaCI:5.0 g/L pH: 7.2±0.2) within 24-hour. The 10-fold serial dilutions were spread on Baird-Parker Agar Medium supplemented with Egg Yolk Enrichment (Becton Dickinson). Typical colonies (dark gray to black colonies with clear zones) were selected and counted for further identification analysis followed by the 24-h for 37 ºC incubation. Phenotypic identification tests including Gram staining, oxidase testing of cytochorome oxidase with indicator (tetramethyl-p-phenylenediamine) conversion to the indophenols catalase (A slide drop with 3% H_2O_2 onto the presumptive *S.aureus* isolates on microscope slides), mannitol fermentation (mannitol-fermentation as a carbohydrate source in the presence of phenol red as a pH indicator to detect mannitol-fermenting Staphylococci), and DNase (DNA hydrolysis test composed of growing microorganism in the DNAse test agar medium that produces Deoxyribonuclease when the DNA is broken down resulting with clear zone and green color fades) were performed. The presumptive (typical colonies) *S. aureus* colonies (n=47) were taken into consideration for further identification analysis.

Genomic DNA Isolation and 16S rRNA Sequencing

The genomic DNA isolation of the presumed *S. aureus* colonies (n=47) was performed by using GeneAll® (South Korea) genomic DNA isolation kit according to the manufacturer`s instructions. Isolated genomic DNA samples were stored at –20 ºC until PCR analysis. The 16S rRNA PCR analysis was performed according to the Frank et al. (2008). The 16S rRNA gene were amplified in a 50 µl reaction volume including 1xPCR buffer (Maximo, GeneON), 0.2 mM of each dNTPs, 2.5 mM $MgCl_2$, and 0.5 µM of each primer (16S rRNA:27F-AGAGTTTGATCCTGGCTCAG and 1492R-GGTTACCTTGTTACGACTT) (Suardana, 2014). The PCR reaction was performed as following conditions: 2 min initial denaturation at 95 °C, 25 cycles of denaturation 1 min at 95 °C, annealing at 55°C for 1 min, and extension at 72°C for 2 min and 10 min final extension at 72°C. The obtained PCR

amplicons (~1465 bp) were evaluated by agarose gel electrophoresis (1.0 %) and screened by a transilluminator implemented in WiseDoc Gel Doc System. The purification of 16S rRNA gene amplicons was performed by BMLabosis (Ankara, Turkey) using the ExoSap-IT (Affymetrix) kit. Later on, samples were sent to Macrogen (Amsterdam, The Netherlands) for the unidirectional sequencing via ABI 3730XL automated sequencer (Applied Biosystems, Foster City, CA, USA), and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The obtained reads were aligned and trimmed using the SILVA (Quast et al., 2013). All 16S rRNA gene sequences (n=47) were deposited into the NCBI GenBank followed by the nucleotide BLAST analysis (NCBI Accession No: MK791580-MK79194 and MN629248-MN629279) (Table 1).

Methicillin susceptibility testing

In order to detect MRSA identified by 16S rRNA sequencing, the agar screening method was performed according to the Brown et al.,2008. Briefly, the density of the *S. aureus* isolates was arranged to the 0.5 McFarland standard. After that, a spot inoculation (10 µl) of *S. aureus* into the Mueller Hinton Agar medium (HiMedia) including 4% NaCI (Conda) and 6 mg/L methicillin (Sigma) was performed. Plates were incubated at 37°C for 24-hour. The growth of any single colonies on methicillin plate is evaluated as resistant (Brown and Yates,1986; Brown et al., 2008).

Results and Discussion

The preparation and consumption of the cheese products with unhygienic conditions could lead to the proliferation of the *S. aureus* in cheese and it can be posing a high risk for public health. Detection, enumeration, and identification of the *S. aureus* especially coagulase positive and methicillin - resistant strains are significant. While coagulase-positive *S. aureus* strains can produce an enterotoxin, coagulase-negative isolates could able to produce enterotoxin (Nunes et al., 2015; Yildirim et al., 2019). Therefore, coagulase - negative *S. aureus* strains should be taken into consideration. In Turkey, there have been several studies that indicate the prevalence and presence of *S. aureus* strains in various cheese samples. The detection percentage were ranging from 20.2% to 92% (Yücel and Anıl, 20011; Gökmen et al., 2013; Bingöl and Toğay, 2017). The high percentage of the detection could indicate health risk in the cheese samples which has been consumed widely in Turkey. In our study, out of 19 white cheese samples, three (n=3) (15%) *S. aureus* were identified and two of them were reported as methicillin-resistant (Table 1). Similarly, the detection percentage of MRSA is not high in Turkey. For example, Saka and Gulel (2018) reported MRSA was 9 %. In another study, the detection percentage was 1.70 %, even though MRSA was investigated from 175 milk and dairy products (Ektik et al., 2017). Nevertheless, these data could show that a serious health problem.

All cheese samples were evaluated quantitatively in this study. The enumeration results were $1.6x10^4$ CFU/g (CE_1), $9.77x10^1$ CFU/g (CE_2), $3.1x10^3$ CFU/g (CE_3), $1.51x10^6$ CFU/g (CE_4), $6.35x10^7$ CFU/g (CE_5), $2.53x10^7$ CFU/g (CE_6), $1.63x10^5$ CFU/g (CE_7), $6.78x10^4$ CFU/g (CE_8), $8.05x10^5$ CFU/g (CE_9), $1.68x10^3$ CFU/g (CE_10), $1.27x10^3$ CFU/g (CE_11), $3.40x10^4$ CFU/g (CE_12), $2.51x10^7$ CFU/g (CE_13), $1.40x10^7$ CFU/g (CE_14), $2.34x10^7$ CFU/g (CE_15), $2.18x0^7$ CFU/g (CE_16), $1.67x0^6$ CFU/g (CE_17), $1.70x0^6$ CFU/g (CE_18), $1.30x10^5$ CFU/g (CE_20). The microbiological criteria in terms of the presence of coagulase-positive *Staphylococcus* species in cheese products established by the Food and Drug Administration (FDA) is 10^2-10^3 CFU/g was acceptable (https://www.fda.gov/media/74723/download). At the same time, Turkish Food Codex Microbiological Criteria takes into consideration the same reliability limits (10^2-10^3 CFU/g) in cheese products (Turkish Official Journal, 2011) However, the presence of *Staphylococcus* species more than 10^4 CFU/gr in cheese product considered to be risky according to the compliance Policy Guide of FDA (Kadiroğlu et al., 2014; https://www.fda.gov/media/74723/download). In this study, 15 out of the 19 cheese samples included more than 10^4 CFU/g presumed *Staphylococcus* species could be considered as hazardous for public health. The number of *Staphylococcus* (CFU) or concentration of enterotoxin can be shown a determining factor of risk situation. In other words, the enterotoxigenic strains of *Staphylococcus* is necessary to grow before the toxin production at detectable levels. Thereby, to cause an infection, a high dose of *Staphylococcus* is required (Food Safety Authority of Ireland 2011; Pollitt et al., 2018).

Table 1. Phenotypic characteristics and 16S rRNA genotypic identification of *S. aureus, S. carnosus, E. faecalis,* and M. *caseolyticus, E. faecium, E.durans,* and *E. gallinarum* isolates obtained from cheese samples

No	ID	16S rRNA	GenBank_ Accession No	Gram_Reaction morphology	O	C	M	D	Methicillin (R/S)
CE_2	CE_2	*Staphylococcus carnosus* CE_2	MK791585	(+)-coccus	(-)	(+)	(+)	(+)	
CE_3	CE_3_2	*Enterococcus faecalis* CE_3_2	MK791587	(+)-coccus	(-)	(+)	(+)	(-)	
CE_1	CE_1_1	*Enterococcus faecalis* CE_1_1	MK791580	(+)-coccus	(-)	(+)	(+)	(+)	
CE_1	CE_1_2	*Enterococcus faecalis* CE_1_2	MK791581	(+)-coccus	(-)	(-)	(+)	(+)	
CE_1	CE_1_3	*Enterococcus faecalis* CE_1_3	MK791582	(+)-coccus	(-)	(+/-)	(+)	(-)	
CE_1	CE_1_4	*Enterococcus faecalis* CE_1_4	MK791583	(+)-coccus	(-)	(+/-)	(+)	(-)	
CE_1	CE_1_5	*Enterococcus faecalis* CE_1_5	MK791584	(+)-coccus	(-)	(+/-)	(+)	(-)	
CE_4	CE_4_2	*Enterococcus faecalis* CE_4_2	MK791592	(+)-coccus	(-)	(+)	(+)	(+)	
CE_4	CE_4_3	*Enterococcus faecalis* CE_4_3	MK791593	(+)-coccus	(-)	(+/-)	(+)	(-)	
CE_4	CE_4_4	*Enterococcus faecalis* CE_4_4	MK791594	(+)-coccus	(-)	(+/-)	(+)	(+)	
CE_3	CE_3_3	*Enterococcus faecalis* CE_3_3	MK791588	(+)-coccus	(-)	(+)	(+)	(+)	
CE_3	CE_3_4	*Staphylococcus aureus* CE_3_4	MK791589	(+)-coccus	(-)	(+)	(-)	(-)	S
CE_3	CE_3_5	*Enterococcus faecalis* CE_3_5	MK791590	(+)-coccus	(-)	(+)	(+)	(+)	
CE_4	CE_4_1	*Enterococcus faecalis* CE_4_1	MK791591	(+)-coccus	(-)	(+)	(+)	(-)	
CE_3	CE_3_1	*Macrococcus caseolyticus* CE_3_1	MK791586	(+)-coccus	(-)	(+)	(-)	(-)	
CE_5	CE_5_1	*Enterococcus faecium* CE_5_1	MN629248	(+)-coccus	(-)	(-)	(-)	(-)	
CE_5	CE_5_3	*Enterococcus durans* CE_5_3	MN629249	(+)-coccus	(-)	(-)	(-)	(-)	
CE_5	CE_5_4	*Enterococcus faecium* CE_5_4	MN629250	(+)-coccus	(+)	(-)	(-)	(-)	
CE_6	CE_6_1	*Enterococcus faecium* CE_6_1	MN629251	(+)-coccus	(-)	(-)	(-)	(-)	
CE_6	CE_6_2	*Enterococcus faecium* CE_6_2	MN629252	(+)-coccus	(-)	(-)	(+)	(-)	
CE_6	CE_6_3	*Enterococcus faecium* CE_6_3	MN629253	(+)-coccus	(+)	(-)	(+)	(-)	
CE_7	CE_7_2	*Enterococcus faecium* CE_7_2	MN629254	(+)-coccus	(-)	(-)	(-)	(-)	
CE_8	CE_8_1	*Enterococcus faecium* CE_8_1	MN629255	(+)-coccus	(+)	(-)	(-)	(-)	

CE_8	CE_8_2	*Enterococcus durans* CE_8_2	MN629256	(+)-coccus	(-)	(-)	(-)	(-)	
CE_8	CE_8_3	*Enterococcus faecium* CE_8_3	MN629257	(+)-coccus	(-)	(-)	(-)	(-)	
CE_9	CE_9_2	*Enterococcus faecium* CE_9_2	MN629258	(+)-coccus	(+)	(-)	(-)	(-)	
CE_10	CE_10_1	*Enterococcus faecium* CE_10_1	MN629259	(+)-coccus	(-)	(-)	(-)	(-)	
CE_10	CE_10_2	*Enterococcus faecium* CE_10_2	MN629260	(+)-coccus	(-)	(-)	(-)	(+)	
CE_11	CE_11_1	*Enterococcus faecium* CE_11_1	MN629261	(+)-coccus	(+)	(-)	(-)	(-)	
CE_11	CE_11_2	*Enterococcus durans* CE_11_2	MN629262	(+)-coccus	(-)	(-)	(-)	(-)	
CE_11	CE_11_3	*Enterococcus durans* CE_11_3	MN629263	(+)-coccus	(+)	(-)	(-)	(-)	
CE_12	CE_12_2	*Enterococcus faecalis* CE_12_2	MN629264	(+)-coccus	(-)	(-)	(+)	(-)	
CE_12	CE_12_3	*Enterococcus faecalis* CE_12_3	MN629265	(+)-coccus	(-)	(-)	(+)	(-)	
CE_12	CE_12_4	*Staphylococcus aureus* CE_12_4	MN629266	(+)-coccus	(-)	(+)	(+)	(+)	R
CE_13	CE_13_1	*Staphylococcus aureus* CE_13_1	MN629267	(+)-coccus	(-)	(+)	(+)	(+)	R
CE_14	CE_14_2	*Enterococcus faecalis* CE_14_2	MN629268	(+)-coccus	(-)	(-)	(+)	(+)	
CE_14	CE_14_3	*Enterococcus faecalis* CE_14_3	MN629269	(+)-coccus	(+)	(-)	(+)	(+)	
CE_15	CE_15_1	*Enterococcus faecalis* CE_15_1	MN629270	(+)-coccus	(+)	(-)	(+)	(+)	
CE_15	CE_15_3	*Enterococcus faecalis* CE_15_3	MN629271	(+)-coccus	(-)	(-)	(+)	(+)	
CE_16	CE_16_2	*Enterococcus faecalis* CE_16_2	MN629272	(+)-coccus	(-)	(-)	(+)	(+)	
CE_17	CE_17_1	*Enterococcus gallinarum* CE_17_1	MN629273	(+)-coccus	(-)	(-)	(+)	(+)	
CE_18	CE_18_1	*Enterococcus faecalis* CE_18_1	MN629274	(+)-coccus	(-)	(-)	(+)	(+)	
CE_18	CE_18_2	*Enterococcus faecalis* CE_18_2	MN629275	(+)-coccus	(+)	(-)	(+)	(+)	
CE_18	CE_18_3	*Enterococcus faecalis* CE_18_3	MN629276	(+)-coccus	(+)	(-)	(+)	(+)	
CE_20	CE_20_1	*Enterococcus faecalis* CE_20_1	MN629277	(+)-coccus	(-)	(-)	(+)	(-)	
CE_20	CE_20_3	*Enterococcus faecalis* CE_20_3	MN629278	(+)-coccus	(-)	(-)	(+)	(-)	
CE_20	CE_20_4	*Enterococcus faecalis* CE_20_4	MN629279	(+)-coccus	(-)	(-)	(+)	(-)	

O:Oxidase, **C**:Catalase, **M**:Mannitol fermentation, **D**:DNAse, **Methicillin:** Methicillin Susceptibility, **S: S**usceptible, **R**: Resistant **(-):** Negative reaction, **(+)** : Positive reaction, **(+/-)** : Late positive

Presumptive *S. aureus* isolates (isolate IDs: CE_12_4 and CE_13_1) were compatible with the phenotypic identification tests including oxidase, catalase, mannitol fermentation, and Dnase. However, presumptive *S. aureus* isolate (ID: CE_3_4) was mannitol fermentation and DNAse tests were negative (Table 1). Although phenotypic tests for the isolate CE_3_4 were not coherent, some of strains of the *S. aureus* could show a negative reaction for the DNase and mannitol fermentation tests (Kateete et al., 2010). According to the 16S rRNA identification results, presumptive isolates (IDs: CE_13_1, CE_12_4 and CE_3_4) were identified as *S. aureus.* In accordance with phenotypic identification tests for the isolates including CE_2, CE_1_1, CE_4_2, CE_4_4, CE_3_3, and CE_3_5 were considered as *S. aureus.* However, the 16S rRNA identification test showed that these isolates were identified as CE_2 (*S. carnosus*), CE_1_1 (*E. faecalis)*, CE_4_2 (*E. faecalis)*, CE_4_4 (*E. faecalis*, CE_3_3 (*E. faecalis)*, and CE_3_5 (*E. faecalis).* Therefore, our results showed that some of the phenotypic identification tests did not correspond to the genotypic identification test. Considering the phenotypic results in Table 1, it was seen that only 47 of the phenotypic test results did not indicate *S. aureus*. On occasion, phenotypic tests can be variable under some conditions. For instance, *E. faecalis* is catalase- positive under the acquisition of heme however, *E. faecalis* strains are catalase negative (Frankkenberg et al., 2002). The 16S rRNA analysis showed that the other *Staphylococaceae* members including *Staphylococcus carnosus* (n=1), and *Macrococcus caseolyticus* (n=1) were reported in this study. Moreover, *Enterococcus faecalis* (n=25) *Enterococcus faecium (n=12), Enterococcus durans (n=4), and Enterococcus gallinarum (n=1)* belonging to the *Enterococcaceae* family was reported in this study (Table 1). Although *E. gallinarum* was reported from clinical samples in Turkey (Özseven et al., 2011), *E. gallinarum* can be isolated during cheese making and ripening procedure. In Italy*, E. gallinarum* was reported a low abundance in artisanal Italian goat's cheese during ripening procedure (Suzzi et al., 2011).

S. carnosus is generally isolated from meat products or fish and it`s known as meat starter culture (Bückle et al., 2017). Similarly, in Turkey, *S. carnosus* was reported from Turkish fermented sausage (Nazli, 1998). Another study that was carried on in France, *S. carnosus* was detected only in dry sausage samples (Coton et al., 2010). The detection of *S. carnosus* in our study could show the contamination of cheese samples. *M. caseolyticus* was also identified in various dairy and meat food sources related to flavor development (Mazhar et al., 2018). Besides, *M. caseolyticus* can be isolated from bovine milk, chicken, and humans. In Switzerland, *M. caseolyticus* was isolated from bovine mastitis milk (Schvendener et al., 2017). However, to best our knowledge*, M. caseolyticus* has not been detected from white cheese samples in Turkey before. As distinct from *S. carnosus* and *M. caseolyticus, E. faecalis* is known as a flora member of the gastrointestinal tract in humans and animals (Abdeen et al., 2016). However, the presence of *E. faecalis* in food sources such as cheese could show fecal contamination and/or inadequate hygienic measures in cheese samples. Moreover, the transmission of *E. faecalis* to the human by consumption of dairy products could cause various infections (Anderson et al., 2016). Similarly, various antibiotic - resistant *Enterococci* such as *E. faecium* has been reported from nosocomial-acquired patients (Sanders et al., 2010). Along with the harmful effects of *Enterococci,* these species are also known to have probiotic potential. Because *Enterococci* has a tolerance to the salts and acids thereby, *Enterococci* could adapt to various foods and could involve the fermentation process of cheese. (Hanchi et al., 2018). And another striking feature of *Enterococci* including *E. faecalis*, *E. faecium*, and *E. durans* has lipolytic activity and production of aromatic compounds (Amaral et al., 2016). In Turkey, *E. faecium* has been used for cheese production as a starter culture. And they were concluded that *E. faecium* FAIR-E 198 could be used as a starter culture (Göncüoglu et al., 2009).

Conclusion

In conclusion, *S. aureus, E. faecalis, E. faecium, E. durans, E. gallinarum*, *S. carnosus*, and *M. caseolyticus* were identified by phenotypic and genotypic identification methodologies. Phenotypic identification tests results should be validated by genotypic identification tests. The detection of MRSA in our study could show the significance of the methicillin resistance in cheese samples for public health. To prevent the transmission of *S. aureus* to cheese products, hygiene and sanitation precautions should be taken during production and sales of the cheese. Also, critical control points should be determined. According to our data, the presence of *S. aureus* and *Enterococci* in cheese products could give an opinion about transmission strategies of these bacteria needed to be studied.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived the conflict of interests.

Ethics committee approval: Author declare that this study does not include any experiments with human or animal subjects.

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