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Investigation of 16S rRNA, *mecA* and *nuc* genes in coagulase-positive and negative Staphylococci by Real-Time PCR

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ABSTRACT

Objective: *Staphylococcus aureus* is a Gram-positive and round-shaped bacterium. It is often positive for catalase and nitrate reduction. Pathogenic isolates support infections by producing protein toxins and the expression of a cell-surface protein virulence factors. Sepsis-related to methicillin-resistant *S. aureus* (MRSA) has significant morbidity and high mortality rates (15-30%). The methicillin resistance for *S. aureus* is coded with the *MecA* gene, while the methicillin sensitivity is coded with the *Nuc* gene, and they are chromosomal. Similarly, it is coded with the coagulase gene for *S. aureus* (*Coa*).

Materials and Methods: In this study, the 16S rRNA gene identification by Real-Time PCR was investigated in forty *S. aureus* isolates, which were cultured at different times in terms of MIC and SIR tests. The isolates used in the study were determined at the gene level in terms of their differences in methicillin resistance gene (*MecA*), methicillin susceptibility gene (*Nuc*), coagulase gene (*Coa*) and intra-species differences were examined.

Results: As a result of the study, *Staphylococcus* spp. yielded positive results with 16S rRNA gene-specific primers in all isolates. Real-Time PCR analysis of the isolates with SYBRGreen-based PCR analysis was performed with 16S rRNA gene-specific primers, and the samples were confirmed to be *Staphylococcus*. Analysis at the family level was followed by *Coa*, *Nuc*, and *MecA* gene Real-Time PCR results, and it was found that, in terms of *Coa* and *Nuc* genes, 19 isolates were positive and 21 isolates were negative. In terms of *MecA* gene, 16 isolates were positive according to the positive sigmoidal curves and to the single peak melting values, whereas 24 isolates were found to be negative.

Conclusion: It is thought that this study will benefit the community by contributing to the rapid and effective treatment and diagnosis of infections caused by coagulase-positive/negative *Staphylococci*.

Keywords: *Coagulase gene, MecA gene, Nuc gene, Staphylococcus aureus, 16S rRNA gene*

INTRODUCTION

S. aureus belongs to the family of *Micrococcaceae* and is grouped under the *Staphylococcus* genus. They are Gram-positive, oxidase-negative, catalase-positive, immobile, non-spore-producing, facultative anaerobe, round coce-shaped microorganisms. As cell division occurs at different planes, the cells are seen together in botryoid form in preparations. *S. aureus* can be produced in the usually used media, at a temperature of 37°C and pH 7.4. They form porcelain-shaped, convex, smooth-surfaced, often yellow-pigmented colonies in the blood gel

medium. Around the colonies, there are generally characteristic hemolysis zones (Levinson, 2002). Some studies carried out in recent years have aimed to increase the resistance of *S. aureus* antibiotics against Methicillin Resistant *Staphylococcus aureus* (MRSA). In studies carried out for this purpose, it has been reported that successful results have been achieved in molecular-based analyzes (Sharaf et al., 2021). In addition, it has been reported that *S. aureus*, the best-known food pathogen, was isolated from waste containers in food businesses and the presence of resistance genes was observed in molecular studies of isolated *S. aureus* isolates (Shahid et al., 2021).

The *Staphylococcus* family's pathogenicity is generally based on the ability to produce coagulase encoded by the *Coa* gene, and coagulase-negative staphylococci are considered secondary infectious pathogens. These bacteria have a huge variety of resistance genes and have more than 40 genes related to resistance in Staphylococci. Some resistance properties are provided by the *MecA* gene (Yadav et al., 2018). The *MecA* gene encodes methicillin resistance, and it is chromosomal. The *MecA* gene is found in all methicillin-resistant coagulase-negative Staphylococci (MRCNS) strains. Strains with this gene are resistant to all beta-lactams because they produce a new penicillin-binding protein (PBP). However, methicillin resistance is not always detectable in routine tests as it is affected by environmental conditions. Therefore, a methicillin-resistant *Staphylococci* can be identified as susceptible. Therefore, PCR is a useful and efficiently method with high sensitivity and specificity in determining methicillin resistance in Staphylococci (Willke et al., 2012). This study aimed at rapid and effective treatment and diagnosis of infections caused by coagulase positive/negative Staphylococci by molecular methods.

MATERIALS and METHODS

Bacterial strains. This study was carried out with the permission of the Local Ethics Committee of Faculty of Medicine, Non-Interventional Clinical Research Ethics Committee on 03.06.2016, and with decision number 09. *Staphylococcus* spp. isolates were isolated from culture samples taken at different times and in various regions in Turkey. Bacterial isolations were carried out on Blood (BA) Agar. Then, they are incubated at 37°C for 18-24 hours. Samples with colony growth were examined macroscopically and microscopically. Colonies suspected to be *Staphylococcus* spp. were identified using standard bacteriological methods (Gram staining, mobility, catalase, lamina, and tube coagulase test, DNase, mannitol fermentation). Accordingly, 20 coagulase-positive and 20 coagulase-negative *Staphylococci* spp. strains obtained as a result of lamina and tube coagulase tests were stored in 15% glycerol Trypticase Soy Broth (TSB) until the time of PCR procedure at -20°C (Bilgehan, 2002; Kaya et al., 2003; Tok and Coşkun, 2010; Nia et al., 2011). Biochemical identifications of the isolated strains, coagulase, and oxacillin MIC tests, and antibiotic susceptibilities were performed using the BD Phoenix™ Bacteria Identification and Antibiogram Device (Becton Dickinson, USA) by ID/AST Combo kit (Tok and Coşkun, 2010; Uçan, 2014). Total DNA isolation was performed using *Staphylococcus* spp. strains identified in routine procedures and extracts obtained in the culture medium. Molecular identification of bacteria was performed by Real-Time PCR for the 16S rRNA gene. For the molecular characterization of isolates, the *MecA* and *Nuc* genes were investigated. Coagulase results (*Coa*) were evaluated depending on *Mec* and *Nuc* gene results. Isolation of bacterial DNA from culture plates was performed with the QIAamp DNA mini kit (Qiagen). The DNA isolations were performed according to the manufacturer's instructions. Molecular steps were carried out with the support of Van Yuzuncu Yil University, Biotechnology Application and Research Center, and Van Yuzuncu Yil University Fisheries Faculty, Fish Disease Laboratories.

Bacterial identification and antibacterial activity. All isolates were individually adjusted to 0.5 optical density (OD). Bacterial suspensions were transferred in the BD Phoenix (Phoenix™ Automated Microbiology System-Becton Dickinson) identification kit. The kit was incubated for 18 hours at 37°C. The samples were evaluated for 26 antibiotics according to their Minimum Inhibition Concentration (MIC) and resistance (SIR) status (Önalan, 2019).

DNA Isolation. DNA isolations were performed using DNA Mini Kit (Qiagen) with QIAcube automatic isolation robot. The Real-Time PCR was carried out 25 µl total volume of specific forward and reverse primers (27F-1492R), SYBR Green-based qPCR Mastermix and water (Önalan and Yavuz, 2019).

Primers. For the identification of *Staphylococcus* spp. isolates, 16S rRNA gene region *Staphylococcus* spp. specific primers were used. The primer set was used as S16-F 5'-AGAGTTTGATCATGGCTCAG-3' and S16-R 5'-GGACTACCAGGGTATCTAAT-3', as reported by Özen et al., (2011). For the methicillin resistance of the isolates, the *MecA* gene, and for the coagulase positivity, the *MecA* gene results were evaluated together with the *Nuc* gene results. *MecA* and *Nuc* gene-specific primer sequences used in the study were used as *MecA*-F-5'-AAAATCGATGGTAAAGGTTGGC-3', *MecA*-R-5'-AGTTCTGCAGTACCGGATTTGC-3', *Nuc*-F-5'-GCGATTGATGGTGATACGGTT and *Nuc*-R 5'-AGCCAAGCCTTGACGAACTAAA-3' (Özen et al., 2011).

Real-Time PCR Analysis. In the Real-Time PCR, pre-denaturation was carried out at 95°C for 10 min. Then, 45 cycles were completed as denaturation at 94°C for 45 sec, annealing at 55°C for 30 sec, and extension at 72°C for 45 sec. Then as the last step, the last-extension process was carried out at 72°C for 7 minutes. Non-template control (NTC) tubes were used as a negative sample control. After analysis, sigmoidal curves have been evaluated as positive (Altinok et al., 2007).

RESULTS

In addition to 40 strains used in the study, *S. aureus* ATCC 25923, *S. aureus* ATCC 6538, and *S. aureus* ATCC 29213 strains were used as the controls for *MecA*, *Nuc*, and *Coa* genes. According to the lamina and tube coagulase test results, 20 samples that were coagulase-positive were identified as *S. aureus* while 20 samples that were coagulase-negative were identified as *Staphylococcus* spp. Ten of the coagulase-negative staphylococci were identified as *S. epidermidis*, 5 were identified as *S. haemolyticus*, 2 were identified as *S. auricularis* and, *S. hominis*, one was identified as *S. lugdunensis*, and one was identified as *S. warneri*. Coagulase results of coagulase-positive and negative *Staphylococci* spp. and oxacillin MIC values were determined in BD Phonenix™ 100 instrument. Accordingly, it was determined that one of the coagulase-positive *S. aureus* strains was Methicillin-Resistant *S. aureus* (MRSA). Methicillin-Resistant Coagulase Negative *Staphylococcus* (MRCNS) was detected in 15 coagulase-negative *Staphylococci* spp. (Table 1).

To determine *Staphylococcus* spp. isolates using molecular methods, PCR was performed with family-specific primers. For this purpose, forward (5'-AGAGTTTGATCATGGCTCAG-3') and reverse (5'-GGACTACCAGGGTATCTAAT-3') primers were used (Özen et al., 2011).

Table 1. Biochemical test results of isolates with methicillin, coagulase, cefoxitin and oxacillin.

No	Isolate name	Coagulase	Cefoxitin MIC	Oxacillin MIC	Methicillin
1	*S. aureus*	+	<=2	<=0.25	S
2	*S. aureus*	+	<=2	0.5	S
3	*S. aureus*	+	4	0.5	S
4	*S. aureus*	+	4	1	S
5	*S. aureus*	+	<=2	<=0.25	S
6	*S. aureus*	+	<=2	<=0.25	S
7	*S. aureus*	+	4	0.5	S
8	*S. aureus*	+	<=2	<=0.25	S
9	*S. aureus*	+	<=2	<=0.25	S
10	*S. aureus*	+	4	0.5	S
11	*S. aureus*	+	4	<=0.25	S
12	*S. aureus*	+	4	0.5	S
13	*S. aureus*	+	<=2	0.5	S
14	*S. aureus*	+	<=2	<=0.25	S
15	*S. aureus*	+	<=2	<=0.25	S
16	*S. aureus*	-	>8	>2	R
17	*S. aureus*	+	<=2	<=0.25	S
18	*S. aureus*	+	<=2	0.5	S
19	*S. aureus*	+	<=2	<=0.25	S
20	*S. aureus*	+	4	0.5	S
21	*S. epidermidis*	-	-	>2	R
22	*S. epidermidis*	-	-	>2	R
23	*S. epidermidis*	-	-	>2	R
24	*S. epidermidis*	-	-	>2	R
25	*S. epidermidis*	-	-	>2	R
26	*S. epidermidis*	-	-	>2	R
27	*S. epidermidis*	-	-	>2	R
28	*S. epidermidis*	-	-	1	R
29	*S. epidermidis*	-	-	<=0.25	S
30	*S. epidermidis*	-	-	<=0.25	S
31	*S. haemolyticus*	-	-	>2	R
32	*S. haemolyticus*	-	-	>2	R
33	*S. haemolyticus*	-	-	>2	R
34	*S. haemolyticus*	-	-	2	R
35	*S. haemolyticus*	-	-	<=0.25	S
36	*S. auricularis*	-	-	>2	R
37	*S. auricularis*	-	-	<=0.25	S
38	*S. hominis*	-	-	2	R
39	*S. lugdunensis*	-	-	>2	R
40	*S. warneri*	-	-	<=0.25	S

*S: Sensitive, R: Resistant, MIC: Minimal Inhibitory Concentration

S. aureus and *S. lugdunensis* with oxacillin MIC values<=2 mcg/mL and cefoxitin MIC values<=4 mcg/mL are mostly methicillin susceptible through

the absence of the *MecA* gene. Coagulase-negative staphylococci (except *S. lugdunensis*) with oxacillin MIC values of>0.25 mcg/mL are mostly methicillin resistant due to the presence of the *MecA* gene.

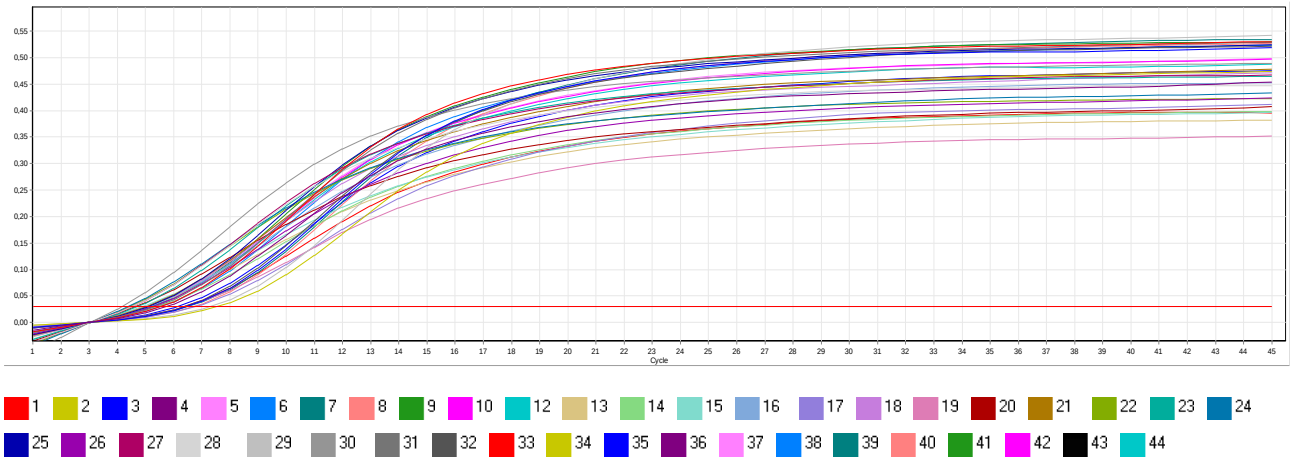

Figure 1. Real-Time PCR results with 16S rRNA gene-specific primers.

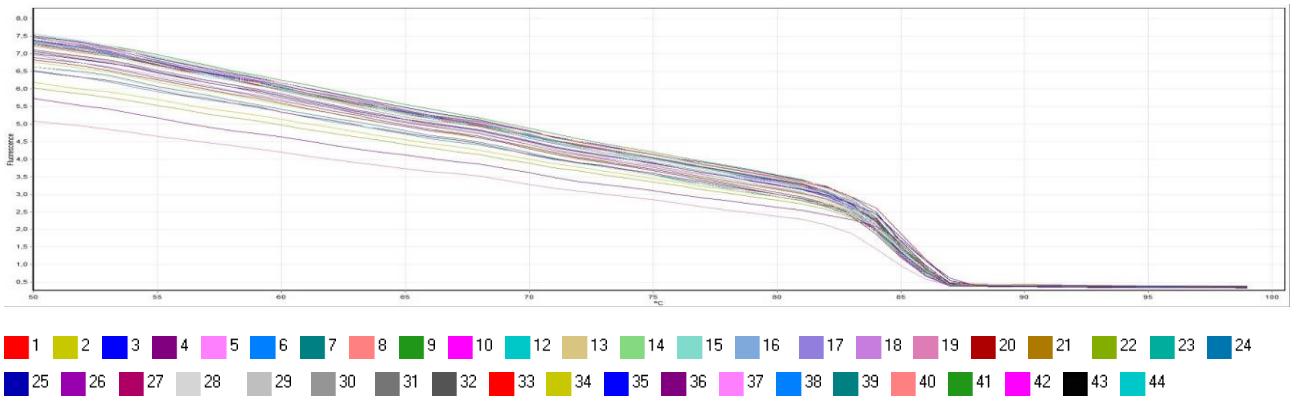

Figure 2. Real-Time PCR melting analysis result of 16S rRNA gene.

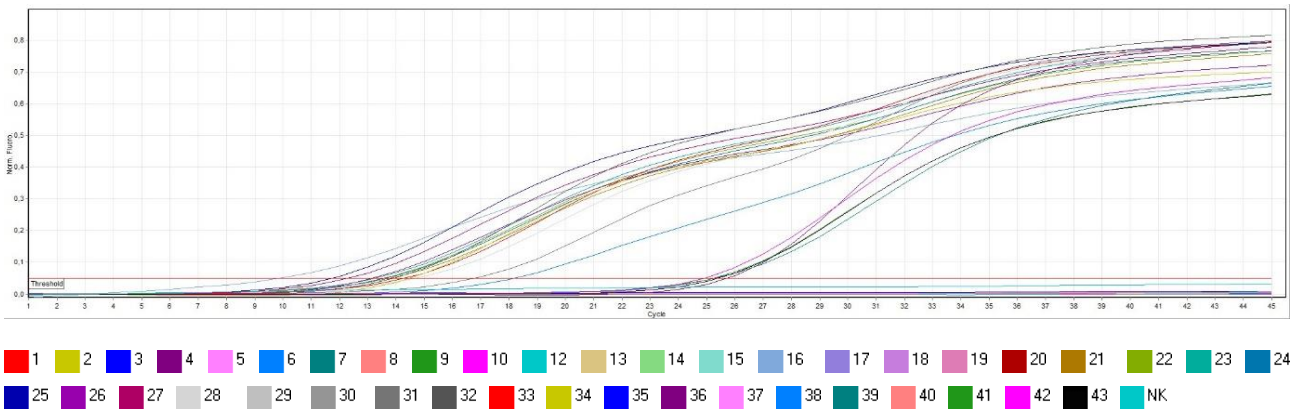

Figure 3. The results of Real-Time PCR with *MecA* gene-specific primers.

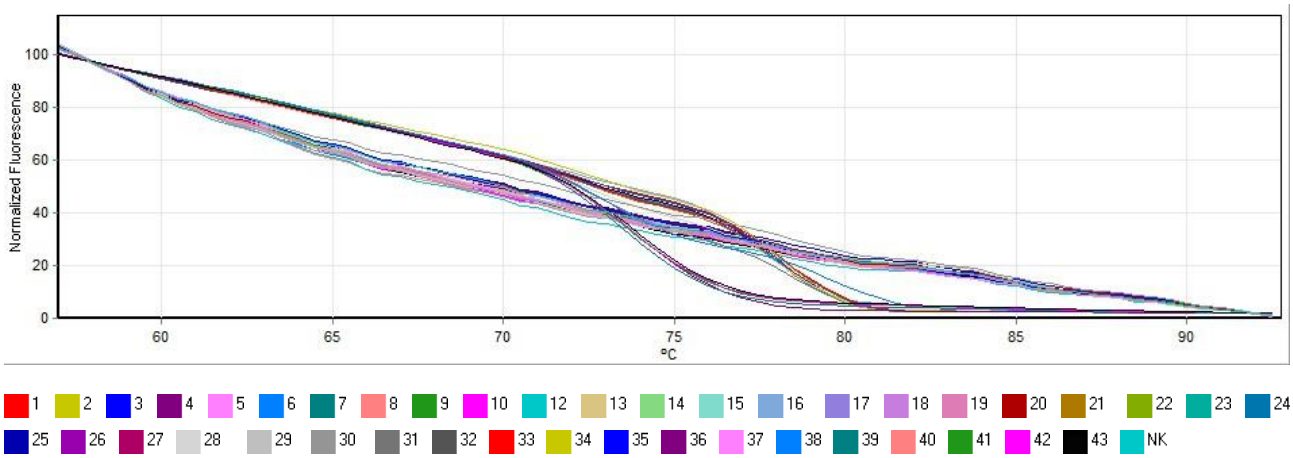

Figure 4. Real-Time PCR melting analysis result of *MecA* gene.

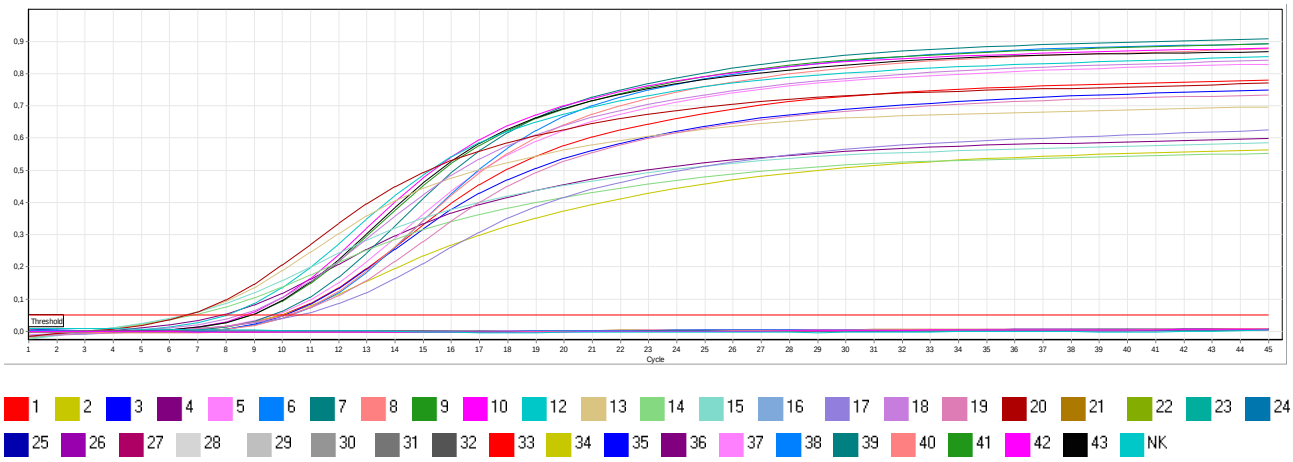

Figure 5. The results of the Real-Time PCR with *Nuc* gene-specific primers.

As a result of the Real-Time PCR procedure using SYBR Green qPCR mastermix (11.0 µl), forward (1.5 µl) and reverse (1.5 µl) primers, DNase, RNase, endotoxin-free water (9.0 µl) and template DNA (2.0 µl), the isolates were determined to be from the *Staphylococcus* family. In the optimized PCR protocol, following the pre-denaturation at 95°C, the 45 cycles performed at 94°C for 30 sec, 57°C for 20 seconds 72°C 30 sec. The final elongation was carried out at 72°C for 5 min to complete the PCR protocol. The results of Real-Time PCR with 16S rRNA gene-specific primers are given below (Figure 1).

After PCR, all strains used in the study showed positive results with 16S rRNA gene-specific primers, and they were identified as *Staphylococcus* spp. (Figure 1). After the Real-Time PCR processing, the accuracy of the study was confirmed by melting analysis. As a result of the melting analysis performed at 55-99°C, all samples started with 5-7.5 fluorescent irradiation and formed melting threshold peaks in the 83-87°C range. Based on these results, primers and PCR amplicon gave a standard melting curve, and all samples used in the study were verified (Figure 2).

Melting analysis showed that PCR amplicons melted in the same range, and similar PCR products were formed. Primers specific to the *MecA* gene (F-5'AAAATCGATGGTAAAGGTTGGC-3' and R-5'AGTTCTGCAGTACCGGATTTGC-3') were used to determine the methicillin resistance of the isolates. It was observed that 18 isolates that gave positive results from PCR were resistant to methicillin (Figure 3).

Accordingly, 18 (16, 21, 22, 23, 24, 25, 26, 27, 28, 31, 32, 33, 34, 36, 38, 39, 41, 42) isolates showed positive results on Real-Time PCR and the gene was evaluated to be positive. After the PCR procedure specific to the *MecA* gene, High-Resolution Melting (HRM) analysis was

performed, and the accuracy of the chemical and the method used in the study was confirmed. High resolution melting graph is given below (Figure 4).

Table 2. 16S rRNA, *Mec A*, *Nuc* and Coagulase gene characteristics of isolates used in this study.

No	Isolate Name	16S rRNA	Coagulase	Nuc gene	MecA gene
1	*S. aureus*	+	+	+	-
2	*S. aureus*	+	+	+	-
3	*S. aureus*	+	+	+	-
4	*S. aureus*	+	+	+	-
5	*S. aureus*	+	+	+	-
6	*S. aureus*	+	+	+	-
7	*S. aureus*	+	+	+	-
8	*S. aureus*	+	+	+	-
9	*S. aureus*	+	+	+	-
10	*S. aureus*	+	+	+	-
11	*S. aureus*	+	+	+	-
12	*S. aureus*	+	+	+	-
13	*S. aureus*	+	+	+	-
14	*S. aureus*	+	+	+	-
15	*S. aureus*	+	+	+	-
16	*S. aureus*	+	+	-	+
17	*S. aureus*	+	+	+	-
18	*S. aureus*	+	+	+	-
19	*S. aureus*	+	+	+	-
20	*S. aureus*	+	+	+	-
21	*S. epidermidis*	+	-	-	+
22	*S. epidermidis*	+	-	-	+
23	*S. epidermidis*	+	-	-	+
24	*S. epidermidis*	+	-	-	+
25	*S. epidermidis*	+	-	-	+
26	*S. epidermidis*	+	-	-	+
27	*S. epidermidis*	+	-	-	+
28	*S. epidermidis*	+	-	-	+
29	*S. epidermidis*	+	-	-	-
30	*S. epidermidis*	+	-	-	-
31	*S. haemolyticus*	+	-	-	+
32	*S. haemolyticus*	+	-	-	+
33	*S. haemolyticus*	+	-	-	+
34	*S. haemolyticus*	+	-	-	+
35	*S. haemolyticus*	+	-	-	-
36	*S. auricularis*	+	-	-	+
37	*S. auricularis*	+	-	-	-
38	*S. hominis*	+	-	-	+
39	*S. lugdunensis*	+	-	-	+
40	*S. warneri*	+	-	-	-
41	*S. aureus* ATCC-25923	+	+	+	+
42	*S. aureus* ATCC-6538	+	+	-	+
43	*S. aureus* ATCC-29213	+	-	+	-

The *Nuc* and *MecA* genes were evaluated together in the method used to determine the coagulase-positive or negative isolates used in the study. Positive and negative results obtained at the end of the PCR analyses using the *Nuc* gene-specific primer sets (F-5'GCGATTGATGGTGATACGGTT and R-5'AGCCAAGCCTTGACGAACTAAA-3') were evaluated together with the *Mec* gene results. The results of the isolates used the *Nuc* gene are given below (Figure 5).

Accordingly, 21 Nuc genes that yielded positive results in Real-Time PCR graph were evaluated as positive. The Real-Time PCR results of 16S rRNA, *Mec A*, *Nuc* and Coagulase results are as follows (Table 2).

According to the SYBR Green-based gene-level Real-Time PCR study results, all isolates were found to be positive for the 16S rRNA gene region-specific primers and were confirmed to be *Staphylococcus*. After the 16S rRNA PCR analysis performed at the family level, the coagulase results were determined together with the Real-Time PCR results for *Nuc* and *MecA* genes. Accordingly, 19 isolates were found to be positive, and 21 isolates were negative in terms of *Coa* and *Nuc* genes. In terms of the *MecA* gene, 16 isolates were positive according to the positive sigmoidal curves and to the single peak melting values, whereas 24 isolates were found to be negative. These results were observed to be in line with the biochemical results.

DISCUSSION

Staphylococcus species are characterized by their biochemical profiles, colony appearance, and hemolytic patterns. These Gram-positive bacteria are biochemically catalase-positive and oxidase-negative, and they use maltose. *S. aureus* produces virulent factors in various types including capsules, enzymes including adhesins, coagulase, catalase, hyaluronidase, staphylokinase, toxins including a toxin, β toxin, δ toxin, leukocidin, enterotoxin, exophilic toxins and toxic shock syndrome toxins causing various diseases in humans and animals (Javid et al., 2018). In a study on methicillin resistance, Seidel et al. (2017) aimed to carry out a rapid and accurate identification of *MecA* and *MecC* genes using nucleic acid lateral flow immunoassay (NALFIA) technology. Examination of 60 identified strains (MRS and non-target bacteria) and 28 methicillin-resistant *S. aureus* (MRSA) isolates from clinical samples was performed by NALFIA, classical PCR-gel electrophoresis and Real-Time PCR and the results were compared. It has been reported that NALFIA was superior to the other methods according to

detection limits, and differentiation between *MecA* and *MecC* can be made by displaying two different alleles on NALFIA test strips.

In this study, biochemical and antimicrobial properties of strains were identified by the BD Phonex ID. Similarly, Nasution et al. (2018) aimed to determine the *MecA* gene and antibiotic resistance pathway in 40 *S. aureus* isolates classified Methicillin-Resistant *S. aureus* (MRSA) by Vitek 2 Compact. The amplification of the *MecA* gene was performed by PCR and showed that all MRSA isolates had a 533 bp *MecA* gene. The antibiotic test of the Vitek 2 Compact showed that, although all isolates were resistant to beta-lactam group antibiotics, they had multiple drug resistance to other common antibiotics such as aminoglycosides, macrolides, and fluoroquinolones. However, the isolates were still susceptible to vancomycin (82.5% isolate), linezolid (97.5% isolate), and tigecycline (100% isolate).

In this study, Real-Time PCR was performed using gene-specific primers. As a result of this process, the isolates used in the study were identified to be from the same family by the 16S rRNA gene. Following this process, Real-Time PCR analyses were performed with *MecA* and *Nuc* genes. As a result, the coagulase properties of isolates were determined according to positive and negative results of *MecA* and *Nuc* genes. The analyses were carried out with *S. aureus, S. epidermidis, S. haemolyticus, S. auricularis, S. hominis, S. lugdunensis,* and *S. warneri* isolates and the reference bacteria (ATCC 25923, ATCC-6538, ATCC-29213). HRM test was used to determine the correct positivity and avoid false-positive results in Real-Time PCR analysis. As a result of this test, the accuracy of the procedures was determined by similar peaks given at ordinary temperatures. Similarly, many studies were conducted adopting identification by PCR. Elhassan et al. (2015) aimed to determine the prevalence of the *MecA* gene using the polymerase chain reaction in methicillin-resistant *S. aureus* (MRSA) isolates and to compare the results with those obtained by the conventional method. In this context, 200 *S. aureus* isolates were taken from patients with different diseases. The phenotypic Kirby-Bauer method, by adopting the E-test, confirmed that methicillin resistance was present in 61.5% of isolates with MICs ranging from 4 μg/mL to 256 μg/mL. Ashraf et al. (2014) aimed to determine *S. aureus*-specific thermonuclease gene (*Nuc*) in chickens by the Real-Time PCR test. The isolated *S. aureus* was susceptible to vancomycin, amoxicillin+clavulanic acid, and cephalothin, respectively, up to 84.5%, 83.8%, and 78.4%, and resistant to ampicillin, oxacillin, and penicillin up to 75%, 73%, and 70.2%, respectively. Hoegh et al. (2014) showed that variations in the *S. aureus*-specific *Nuc* gene could lead to misidentification of methicillin-sensitive and resistant *S. aureus*. Accordingly, in 10 *S. aureus* isolates, Real-Time PCR was performed with primers and probes that were designed explicitly for *Nuc* and *MecA* genes, and a DNA sequence analysis was performed. Hamidi et al. (2015) aimed to determine the prevalence of the production of coagulase (*Coa*) and thermonuclease (*Nuc*) genes and Staphylococcal enterotoxin A (*Sea*) among *S. aureus* samples isolated from various sources. In total, 100 *S. aureus* were isolated from 40 humans, 30 animals, and 30 food samples, and *Coa*, *Nuc*, and *Sea* genes were evaluated by the PCR. According to the culture results, Willke et al. (2012) have reported that of the 48 staphylococci strains, 15 were methicillin-resistant coagulase-negative Staphylococci (MRCNS), 4 were methicillin-resistant *S. aureus* (MRSA), and 14 were methicillin-sensitive coagulase-negative Staphylococci (MSCNS) and 15 were methicillin-sensitive *S. aureus* (MSSA). According to the PCR results, 17 of the strains were found to be MRCNS, 8 were found to be MRSA, 10 were found to be MSCNS and 13 were found to be MSSA. Levi and Towner (2003) have reported that 17 of the 200 blood cultures suspected of containing *Staphylococcus* were found to be positive by PCR, and 16 were found to be positive by culture method.

CONCLUSION

As a result of this study, the results of Real-Time PCR and BD Phoenix ID automatic devices were compared, and were found to be in agreement. It was thought that this study would benefit the community by contributing to the rapid and effective diagnosis and treatment of infections caused by coagulase-positive and negative Staphylococci.

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Author's Contributions: AA and ŞŞO designed the study. AA and ŞŞO performed surgeries. ŞÖ performed molecular analysis from bacteria. AA and ŞÖ performed statistical analysis. AA and ŞÖ participated in drafting and revising the manuscript. AA: Abdulbaki Aksakal, ŞÖ: Şükrü Önalan, ŞŞO: Şeyda Şilan Okalin

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