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Investigation of biofilm formation in methicillin resistant *Staphylococcus aureus* isolates by genotypic and phenotypic methods and effect of vancomycin and teicoplanin on biofilm inhibition

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Abstract: The pathogenicity factors of *Staphylococcus aureus* include biofilm production. In this study, the biofilm production abilities of methicillin-resistant *S. aureus* (MRSA) strains were investigated using genotypic and phenotypic methods. Additionally, the effect of glycopeptides on biofilm was examined. This study included 130 MRSA isolates. Biofilm was detected by the microtiter plate method. The minimum inhibitory concentration (MIC) of glycopeptides was evaluated through the broth microdilution method. The biofilm inhibitor concentration (BIC) values were investigated in isolates with strong biofilm production. The *mecA* (methicillin resistance gene), *icaA,* and *icaD* (biofilm-associated genes) were amplified by polymerase chain reaction techniques. Eighty-one isolates (62.31%) formed biofilms, while thirty isolates (23.08%) exhibited strong biofilm formation. Thirty isolates had higher BIC_{90} values than MIC_{90} values. The *mecA* gene was confirmed in 125 (96.15%) isolates, the *icaA* gene in 96 (73.85%) isolates, and the *icaD* gene in 100 (76.92%) isolates. There was statistical significance between *ica* genes and the biofilm produced ($p<0.05$). In conclusion, increased biofilm formation due to the effect of *ica* genes increases the concentration values at which antibiotics act.

Keywords: Biofilm, polymerase chain reaction, MRSA, antimicrobial resistance.

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INTRODUCTION

Staphylococcus aureus colonizes the mucous membranes and skin of healthy individuals. It can cause a wide range of clinical manifestations, from mild to severe systemic infections. Toxins, enzymes, adherence factors, and biofilm formation are responsible for its pathogenicity (Jin *et al*., 2021). Biofilms consist of groups of microorganisms that attach to either living tissues or inanimate surfaces. This adherence significantly contributes to the organism's resilience against treatments. It increases resistance to antimicrobial treatments and causes permanent infections that are challenging to manage. This heightened resistance is especially significant, as bacteria within biofilms are recognized to be 100 to 100,000 times less susceptible to antimicrobials (Roy *et al.,* 2018; Erdoğmuş and Konak, 2020). When biofilm formation is combined with antibiotic resistance, treating infections becomes increasingly difficult and often results in chronic disease (Sharma *et al*., 2023). Biofilm formation contributes to antimicrobial resistance through various mechanisms. These mechanisms include the low growth rate of bacteria embedded in biofilms, the adverse effects of microenvironmental factors on antimicrobial efficacy, and the challenges associated with antimicrobial diffusion in the presence of biofilms (Rodis *et al.,* 2020). The *icaADBC* operon is responsible for biofilm formation in staphylococci. The *icaADBC* operon catalyzes the synthesis of poly-N-acetyl-beta-1-6-glucosamine (PNAG) oligomers and encodes the polysaccharide intercellular adhesin (PIA) protein. Particularly, the *icaA* and *icaD* genes have a significant impact on biofilm formation in *S. aureus*. While *icaA* shows limited transferase activity on its own, its activity is increased by *icaD* (Avila-Novoa *et al*., 2021; El-Sawaf *et al.,* 2022). When biofilm-related infections are combined with methicillin resistance, it is challenging to establish an appropriate treatment protocol. This study may contribute new data to the existing literature on biofilm and antimicrobial resistance, aiding in the development of treatment strategies. In our study, we examine biofilm development by methicillin-resistant *S. aureus* (MRSA) strains using the microtiter plate method, visualize biofilm production with scanning electron microscopy (SEM), and assess the minimum inhibitory concentration (MIC) and biofilm inhibitory concentration (BIC) values of glycopeptide antibiotics in these isolates. Additionally, the presence of *mecA*, *icaA, and icaD* genes is investigated using polymerase chain reaction (PCR).

MATERIALS AND METHODS

Identification of bacteria and determination of antimicrobial susceptibility

This study included 130 MRSA isolates submitted to the laboratory from April 2020 to December 2022. Bacterial identification and antibiotic susceptibility testing were performed using the Vitek MS system (BioMérieux, France) and the Vitek 2 Compact system (BioMérieux, France). The cefoxitin (30µg) disk diffusion method was

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employed to assess methicillin resistance. The results were interpreted according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (EUCAST, 2023).

Determination of biofilm activity

Biofilm-forming ability was detected by the microtiter plate method as previously described by Christensen *et al* (Chr□stensen *et al.,* 1985). Biofilm formation was evaluated based on optical density (OD) values (Chusr□ *et al.,* 2012). OD values were quantitatively measured at a wavelength of 492 nm using an ELISA reader (ELISA Reader, Chromate Awareness Technology, USA) and interpreted according to the "biofilm formation activity evaluation scale" proposed by Chusri *et al* (Chusr□ *et al.,* 2012). The negative control was *S. aureus* ATCC 29213. *S. aureus* ATCC 25923 (a strong biofilm former) was the positive control. Experiments were performed in triplicate.

Imaging of biofilm production using Scanning Electron Microscope (SEM)

Two randomly selected isolates, one biofilm-forming and the other non-biofilm-forming, were imaged using a SEM. Slide pieces were added to a 12-well plate. Following the formation of biofilms on slide pieces, the slide pieces within the plate were subjected to a washing process and subsequently transferred to a new plate. The wells were filled with 4% glutaraldehyde and left for 60 minutes. Following this, each concentration of ethyl alcohol (70%, 80%, 90%, 96% and pure) was applied separately and left for 10 minutes. Then, the wells were aspirated, and the slide pieces were left to dry. The dried slide pieces were coated with gold/palladium (Au/Pd). After this process, they were seen through a scanning electron microscope. (JEOL, USA) at magnifications of 1000x, 3000x, 5000x and 10000x (Ünsal *et al.,* 2017).

Determination of vancomycin and teicoplanin MIC values

The MIC values of vancomycin (Cayman Chemical Company, USA) and teicoplanin (Sigma-Aldrich, USA) for all isolates were established using the broth microdilution method (CLSI, 2020), with a MIC range of 0.03125-16 µg/mL. The results were interpreted according to the recommendations of the EUCAST (EUCAST, 2023). MIC values higher than 2 µg/mL were considered "resistant", while those equal to or less than 2 µg/mL were deemed "susceptible".

Determination of the antibiofilm effect of vancomycin and teicoplanin

The effect of glycopeptides on the biofilm developed at this stage was evaluated. Isolates that produced strong biofilms were utilized in the study. The isolates were incubated in Trypticase Soy Broth (TSB) with 0.25% glucose for 24 hours at 37°C. Subsequently, the suspensions were diluted at a ratio of 1:20, and 200 µL was transferred into 96-well microplates. Sterile glass beads (Isolab, Turkey), 5-6 mm in diameter, were placed into the wells. The plates were incubated at 37°C for 24 hours to allow biofilm formation on the beads. Serial dilutions (4-4096 µg/mL) of vancomycin and teicoplanin were made in cation-adjusted Mueller-Hinton Broth (CAMHB) in another microplate. Glass beads were added to the wells of the microplate containing antibiotic dilution. It was allowed to incubate at 37°C for 24 hours. Following incubation, the beads were transferred to capped tubes containing 200 µL of CAMHB and vortexed for 5 minutes. Subsequently, 100 µL of the supernatant was transferred to the plates containing 100 µL of CAMHB in their wells. It was allowed to incubate for one day at 37°C. The lowest concentration value at which no growth occurred was recorded as biofilm inhibitory concentration (BIC). Experiments were performed in triplicate (M□lletl□Sezg□n *et al.,* 2019).

Molecular detection of the methicillin resistance gene and biofilm-associated genes

DNA isolation was performed using the boiling method (Tshabalala *et al.,* 2021), and the *mecA, icaA,* and *icaD* genes were investigated using an in-house PCR method with an automated thermal cycler. The primers used were determined after the literature review (Vasudevan *et al.,* 2003; McClure *et al.,* 2006). The method used by McClure *et al*. (McClure *et al.,* 2006) for the investigation of the *mecA* gene region was applied, and the positive control was the *S. aureus* ATCC 43300 strain. Amplification of the *mecA* gene includes denaturation at 94°C for 10 minutes, followed by 30 cycles consisting of denaturation at 94°C for 45 seconds, annealing of primers at 55°C for 45 seconds, and elongation at 72°C for 75 seconds, followed by 10 minutes of final extension at 72°C. For the *icaA* and *icaD* gene regions, the method described by Vasudevan *et al*. (Vasudevan *et al.,* 2003) was applied, using *S. epidermidis* ATCC 35984 as the positive control. Amplification of the *icaA* gene includes denaturation at 95°C for 3 minutes, 40 cycles consisting of denaturation at 95°C for 30 seconds, annealing of primers at 50°C for 30 seconds, and elongation at 72°C for 90 seconds, followed by 15 minutes of final extension at 72°C. Amplification of the *icaD* gene includes denaturation at 92°C for 3 minutes, 30 cycles consisting of denaturation at 92°C for 45 seconds, annealing of primers at 49°C for 45 seconds, and elongation at 72°C for 60 seconds, followed by 7 minutes of final extension at 72°C. Electrophoresis of PCR products was performed using a 2% agarose gel, and the DNA bands from samples were compared with the "GeneON 100 bp Plus Blue DNA ladder" (GeneON, Germany) DNA marker and examined. The presence of bands with sizes of 310 bp for *mecA*, 1315 bp for *icaA,* and 381 bp for *icaD* was investigated (Vasudevan *et al.,* 2003; McClure *et al.,* 2006).

Ethical approval

Approval for this research was granted by the Ondokuz Mayıs University Clinical Research Ethics Committee on 26/10/2022, with approval number 2022/466.

Table 1: Biofilm formation profiles according to clinical specimen types

Sample Type	Number of Samples Forming Biofilm n (%)	Number of Samples Forming Strong Biofilm n (%)
Blood (n=40)	25 (62.5)	9 (22.5)
Catheter Blood (n=40)	15 (37.5)	10 (25)
Exudates (n=50)	41 (82)	11 (22)
Total (n=130)	81 (62.31)	30 (23.08)

Table 2: Antimicrobial resistance profiles of biofilm-forming and non-forming isolates according to clinical specimen types

Antimicrobials	Blood (n =40)		Catheter Blood (n=40)		Exudates (n=50)	
	Biofilm-forming isolates n=25 (%)	Non-biofilm-forming isolates n=15 (%)	Biofilm-forming isolates n=15 (%)	Non-biofilm-forming isolates n=25 (%)	Biofilm-forming isolates n=41 (%)	Non-biofilm-forming isolates n=9 (%)
P	25 (100)	15 (100)	15 (100)	25 (100)	41 (100)	9 (100)
OXA	25 (100)	15 (100)	15 (100)	25 (100)	41 (100)	9 (100)
CIP	5 (20)	2 (13.33)	6 (40)	-	12 (29.27)	4 (44.44)
GN*	-	-	-	-	-	-
VA	-	-	-	-	-	-
TEC	-	-	-	-	-	-
E	11 (44)	2 (13.33)	11 (73.33)	2 (8)	15 (36.58)	1 (11.11)
DA	11 (44)	2 (13.33)	11 (73.33)	2 (8)	15 (36.58)	1 (11.11)
TE	13 (52)	1 (6.67)	12 (80)	-	18 (43.9)	2 (22.22)
LNZ	-	-	-	-	-	-
SXT	1 (4)	1 (6.67)	1 (6.67)	-	2 (4.88)	1 (11.11)
Total	25 (100)	15 (100)	15 (100)	25 (100)	41 (100)	9 (100)

*Susceptibility data for gentamicin are available for 68 isolates, all of which were sensitive to gentamicin. Susceptibility information for the remaining isolates is not available in our data.
P: Penicillin, OXA: Oxacillin, CIP: Ciprofloxacin, GN: Gentamicin, VA: Vancomycin, TEC: Teicoplanin, E: Erythromycin, DA: Clindamycin, TE: Tetracycline, LNZ: Linezolid, SXT: Trimethoprim-sulfamethoxazole

Table 3: MIC_{50}, MIC_{90}, BIC_{50}, BIC_{90}, and MIC value ranges, BIC value ranges for vancomycin and teicoplanin in the isolates

	Glycopeptides	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	BIC_{50} (µg/mL)	BIC_{90} (µg/mL)	MIC Value Ranges (µg/mL)	BIC Value Ranges (µg/mL)
All Isolates (n=130)	Vancomycin	1	1	-	-	0.5-2	-
	Teicoplanin	1	2	-	-	0.25-2	-
Strong Biofilm-Forming Isolates (n=30)	Vancomycin	1	1	256	512	0.5-1	64-4096
	Teicoplanin	1	2	256	1024	0.5-2	32-4096

MIC: Minimum inhibitory concentration, BIC: Biofilm inhibitor concentration

STATISTICAL ANALYSIS

SPSS software version 21 was used to perform the statistical analyses by the Chi-square test. The criterion for accepting a statistically significant result is that the p-value is less than 0.05.

RESULTS

The study included 40 (30.77%) blood cultures (bloodstream infections), 40 (30.77%) catheter blood cultures (catheter-related bloodstream infections), and 50 (38.46%) exudate cultures. Upon analysis of biofilm formation, 81 (62.31%) isolates were found to produce biofilm, while 30 (23.08%) isolates exhibited strong biofilm formation. The biofilm formation profiles of the isolates based on the type of clinical specimens are presented in table I. The images obtained using SEM are presented in fig. 1. SEM analysis revealed no biofilm production in *S. aureus* isolate number 112 (A, B, C), whereas biofilm production was detected in *S. aureus* isolate number 118 (D, E, F). The antimicrobial resistance profiles of biofilm-forming and non-forming isolates according to clinical specimen types are presented in table II.

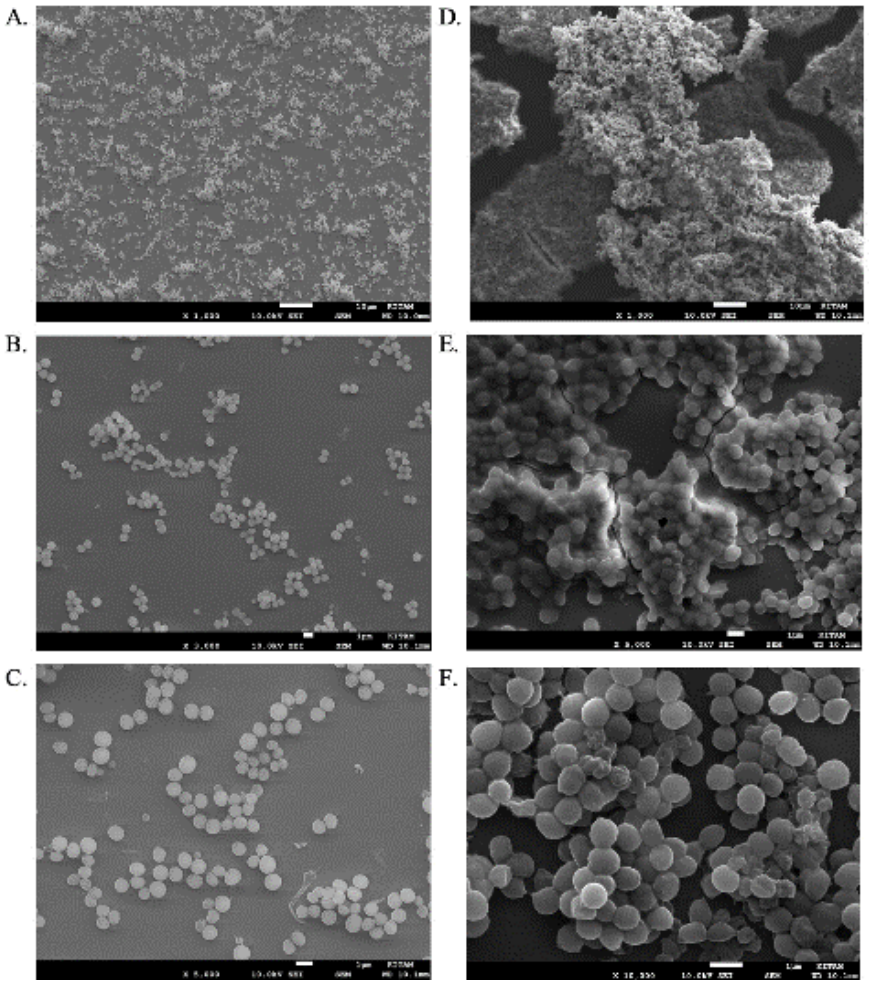
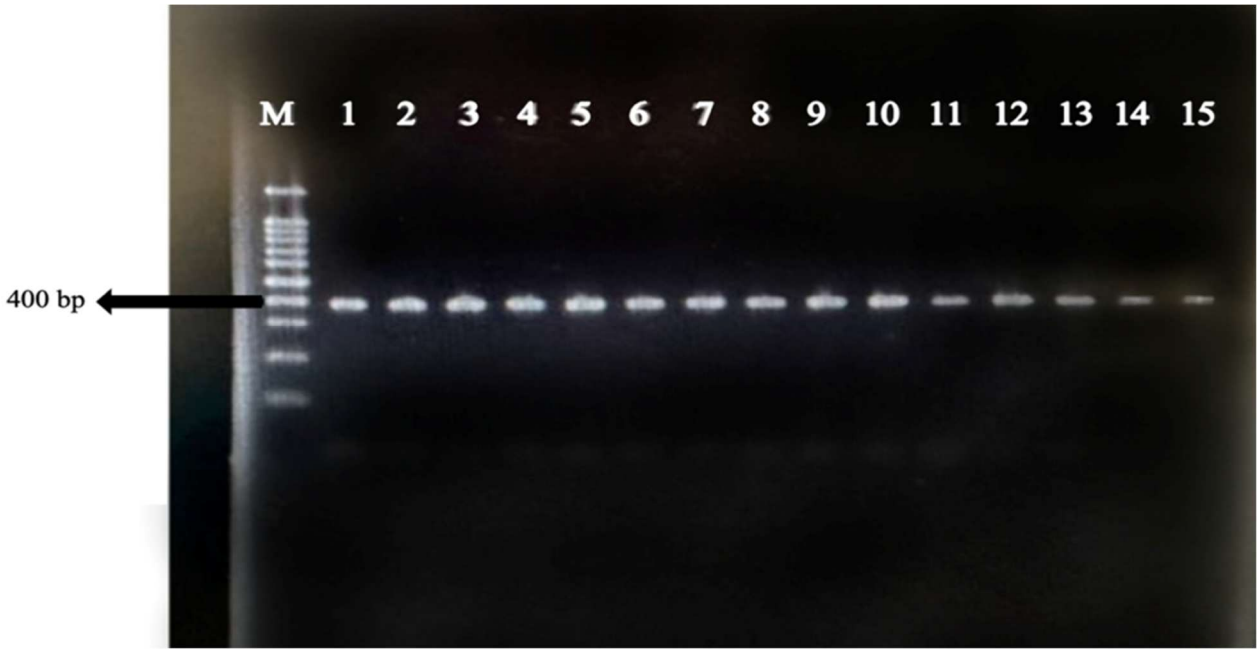


Fig. 1: SEM images of *S. aureus* isolate number 112, which does not produce biofilm (A, B, C) and *S. aureus* isolate number 118, which produces biofilm (D, E, F) (A: 1000x magnification; B: 3000x magnification; C: 5000x magnification; D: 1000x magnification; E: 5000x magnification; F: 10000x magnification)

(M: Marker; 1: *mecA* positive control; 2-15: *mecA* positive isolates; *mecA*:310 bp)
Fig. 2: Gel image of *mecA* positive isolates

(M: Marker; 1, 3, 8, 12, 14: *icaA* negative isolates; 2, 4, 5, 6, 7, 9, 10, 11, 13: *icaA* positive isolates; 15: *icaA* positive control; *icaA*: 1315 bp)

Fig. 3: Gel image of *icaA* positive isolates



(M: Marker; 1: *icaD* positive control; 2-15: *icaD* positive isolates; *icaD*: 385 bp)

Fig. 4: Gel image of *icaD* positive isolates

All isolates were susceptible to glycopeptides based on MIC results. The MIC_{50} and MIC_{90} values represent the minimum concentrations required to inhibit the growth 50% and 90% of the isolates, respectively. Similarly, the BIC_{50} and BIC_{90} values are indicative of the minimum concentrations required to inhibit biofilm formation in 50% and 90% of the isolates, respectively. The MIC_{50}, MIC_{90}, BIC_{50}, and BIC_{90} values, as well as the MIC and BIC value ranges for vancomycin and teicoplanin, are presented in table III. The BIC_{90} values for vancomycin and teicoplanin in the 30 strong biofilm-forming isolates were found to be 512 times higher than their respective MIC_{90} values.

PCR analysis detected the *mecA* gene in 125 (96.15%) of the isolates. Among 81 biofilm-forming isolates, *icaA* and *icaD* genes were observed in 78 (96.3%) of them. In

contrast, among the 49 non-biofilm-forming isolates, the *icaA* gene was detected in 18 (36.73%) and the *icaD* gene was observed in 22 (44.9%) of them. The *icaA* and *icaD* genes were observed in all strong biofilm-forming isolates. A significant relationship was observed between biofilm formation and the expression of the *icaA* and *icaD* genes ($p<0.05$). The band profiles of the genes are visually presented in fig. 2, 3, and 4.

DISCUSSION

In infections associated with biofilm, the biofilm matrix confers resistance to both the host immune system and the effects of antimicrobial agents. (Grande *et al.,* 2020). In order to combat these infections effectively, it is essential to identify biofilm formation, gain an insight into the relationship between biofilm and resistance, and develop new treatment options. In the literature, one of the methods used to measure biofilm formation is the microtiter plate method. SEM is an imaging method that is capable of obtaining high-resolution images (Temel and Eraç, 2018). In our study, two randomly selected isolates, one biofilm-forming and the other non-biofilm-forming, were imaged using SEM, providing evidence of biofilm formation and allowing for a qualitative analysis.

In studies, the ability of *S. aureus* to produce biofilms has been investigated based on specimen types. In a study performed in our country, biofilm formation was detected in 70.5% of S. *aureus* isolates from chronic wound infections (Demir *et al.,* 2020). Hortaç İştar *et al*. (Hortaç İştar *et al.,* 2020) observed that among 83 isolates, wound isolates produced biofilm at a higher rate compared to blood and catheter isolates using the modified Christensen method. In our study, similar to the findings of Hortaç İştar *et al*., exudate isolates were observed to produce higher rates of biofilm than others. This result suggests that in wound infections, the loss of the barrier effect of the skin and disruption of the microflora may have a facilitating effect on biofilm formation.

It is known that biofilm-producing strains have higher antibiotic resistance. İbrahim *et al*. (İbrahim *et al*., 2022) reported significantly higher biofilm formation in MRSA isolates compared to methicillin-sensitive strains, while Gür Vural *et al*. (Gür Vural *et al.,* 2023) found a rate of 68% in MRSA isolates. In agreement with other previous studies, we found that 62.31% of MRSA isolates produced biofilm. Several studies have reported that biofilm-producing isolates exhibit increased resistance to antimicrobials (Demir and Çetik Yıldız, 2020; İbrahim *et al*., 2022). Neopane *et al*. (Neopane *et al.,* 2018) found that biofilm-producing isolates exhibited higher resistance to the antibiotics erythromycin, clindamycin, trimethoprim-sulfamethoxazole, ciprofloxacin, and tetracycline. Similarly, in our study, biofilm-producing isolates exhibited higher resistance to the antibiotics erythromycin, clindamycin, tetracycline, and ciprofloxacin. These results indicate that MRSA isolates have a higher ability to produce biofilm compared to MSSA and that biofilm provides resistance against antibacterial treatments. In our country and international studies on *S. aureus* infections, low resistance rates to trimethoprim-sulfamethoxazole have been reported (Vicett Miguel *et al.,* 2019; Şanlı *et al.,* 2021). In our study, low resistance rates to trimethoprim-sulfamethoxazole were detected in both groups of biofilm-forming and non-biofilm-forming isolates. Resistance to trimethoprim-sulfamethoxazole was not associated with biofilm formation. There are six isolates resistant to trimethoprim-sulfamethoxazole, of which four were found to produce biofilm (two from exudate samples, one from blood, and one from catheter blood samples).

Glycopeptides are the primary antibiotics used to treat MRSA infections. In our study, the MIC_{50} and MIC_{90} values for vancomycin were found to be 1 µg/mL. The MIC_{50} value for teicoplanin was found to be 1 µg/mL, and the MIC_{90} values were found to be 2 µg/mL. Erdoğmuş and Konak (Erdoğmuş and Konak, 2020) demonstrated that the BIC values of vancomycin against biofilm-forming *S. aureus* were higher than the MIC values effective against sessile forms. Nishimura *et al*. (Nishimura *et al.,* 2006) reported six staphylococcal strains obtained from arthroplasty patients. They found the MIC values measured ranging 0.5 and 1 µg/mL and BIC values exceeding 512 µg/mL. In our study, in 30 of the isolates, the BIC_{90} values were observed to be 512 times higher than the MIC_{90} values for vancomycin and teicoplanin. Our results indicate that biofilm reduces antibiotic efficacy and that inhibiting biofilm-forming bacteria is challenging. These bacteria require antibiotic doses that exceed the MIC values.

The impact of the *ica* genes on biofilm production by *S. aureus* is significant. These genes encode enzymes that use UDP-N-acetylglucosamine to catalyse the synthesis of oligosaccharides. The *icaA* gene alone exhibits a low N-acetylglucosamine transferase activity. The enzyme activity of the *icaA* gene increases in the presence of the *icaD* gene (El-Sawaf *et al.,* 2022). Şahin *et al*. (Şahin and Kalelİ, 2018) reported that 89.5% of 152 biofilm-producing *Staphylococcus aureus* isolates contained the *icaA* and *icaD* gene regions. Milletli Sezgin *et al*. (Milletli Sezgin *et al.,* 2019) obtained 86 biofilm-producing *S. aureus* isolates from nasal swab samples. They detected the *icaA* gene in 90.6% and the *icaD* gene in 91.8% of these isolates. As with the studies previously reported on this topic, in our study, the *icaA* gene was found in 96 (73.85%) isolates, while the *icaD* gene was identified in 100 (76.92%) isolates. In 78 (96.30%) of the 81 biofilm-producing isolates, the *icaA* and *icaD* gene regions were found to be positive. Additionally, all 30 isolates with strong biofilm production were positive for these gene regions. The data obtained indicate the significance of *ica* genes in biofilm production. However, the presence of *ica* genes does not always result in biofilm production.

CONCLUSION

The resistance developed by biofilm layers against antimicrobials leads to treatment failures. It is essential to determine and eliminate the biofilm formation potential of bacteria that are responsible for colonization or infection. This strategy will contribute to lowering infection rates, as well as the associated health complications and death. Our study demonstrated that the biofilm formation rates of MRSA strains are high and highlighted the prevalence of biofilm-associated genes. Screening the biofilm formation potential of *S. aureus* bacteria is essential. Identifying the presence of the *ica* genes is essential to enable the timely administration of an effective treatment. As a limitation of our study is that clonal typing could not be performed on the *S. aureus* isolates.

Conflict of interest

The authors declare that they have no conflicts of interest concerning this article.

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